Queen Mary
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Investigating The Molecular Mechanisms Of The Metabolic Control Of T Cell Migration and Functions

Lactate controls pro-inflammatory T cell migration and function

Submitted in partial fulfillment of the requirements of the Degree of Doctor of Philosophy

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For my parents

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STATEMENT OF ORIGINALITY

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Details of collaboration and publications:

In-vivo peritonitis model and peritoneal recruitment (Figure 12 B – F) — *Joanne Smith*

Human blood sampling and cytokine staining (Figure 11E) — *Suchita Nadkarni*

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ABSTRACT

Lactate has long been considered a "waste"-by-product of cellular metabolism, and it is known to accumulate in inflammatory sites. Recent findings have suggested lactate as signaling metabolite, yet its effects on immune cells during inflammation are largely unexplored. This project hence aimed to examine the effects of extracellular lactate on the function of T cells.

I found that the elevated concentration of lactate present in synovia of rheumatoid arthritis (RA) patients inhibits the migration of $CD4^+$ and $CD8^+$ T cells, mediated via the subtype specific transporters Slc5a12 and Slc16a1, respectively. In addition, lactate decreases the cytolytic activity in $CD8^+$ and induces the production of IL-17 in $CD4^+$ T cells.

Intracellular, lactate causes a change in redox state, the increase of TCA-cycle metabolites, the production of ROS and a drop in ATP levels. Concomitantly, I found a decrease of glycolysis and oxidative phosphorylation with no effect on fatty acid metabolism. This metabolic inhibition caused an adaptation attempt to rescue the glycolytic flux due to the relocation of HK1 to the mitochondrial membrane.

I further show that the lactate-mediated reduction of glycolysis is the cause for the observed migratory inhibition in $CD4^+$ T cells, possibly mediated by the NAD^+-dependent enzymes SIRT1 and PARP1. Similarly, I found the lactate-induced IL-17 production to be dependent on NAD metabolism, fatty acid synthesis and FOXO1.

Finally, I could causatively link lactate signaling to chronic T cell infiltrates in RA, as I show that the expression of lactate transporters correlates with the clinical T cell score in the synovia of RA patients. Pharmacological or antibody-mediated blockade of subtype-specific lactate transporters on T cells results in their release from the inflammatory site in a model of peritonitis.

These findings establish lactate as active signaling metabolite that contributes to the perpetuation of chronic inflammation and provide novel therapeutic approaches to combat chronic inflammatory diseases.

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1 ABBREVIATIONS

11βhsd1	11Beta-hydroxysteroid dehydrogenase type 1
2-DG	2-Deoxyglucose
ACC	acetyl-CoA Carboxylase
Akt / PkB	Protein Kinase B
AMPK	5'-AMP activated protein kinase
ANLS	Astrocyte neuron lactate shuttle
AnxA1	Annexin A1
APC	Antigen Presenting Cell
Arg2	Arginase 2
ATP	Adenosine tri phosphate
BBS	Bis Buffered Saline
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium Chloride
cAMP	Cyclic Adenosine monophsophate
CCR/L	-CC- motif receptor / ligand
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CHC	α-cyano-4-hydroxycinnamate
CIDs	Chronic inflammatory Diseases
CO2	Carbon dioxide
CPT1a/2	Carntine palytoyl transferase 1a/2
CTL	Cytotoxic T lymphocyte
CXCR/L	-CXC-motif receptor / ligand
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
DDAO	7-Hydroxy-9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DUBA	Deubiquitinase
EAE	Experimental autoimmune encephalomyelitis
EC	Endothelial cell
EC50	Half maximal effective concentration
ECAR	Extracellular acidification rate
ELISA	Enzyme linked immunosorbent assay

ERK	Extracellular Signal-regulated kinase
ETC	Electron transport chain
FACS	Fluorescent activated cell sorting
Fak	Focal adhesion kinase
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
Fbp1	Fructose-Bisphosphatase 1
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FSC	Forward scatter
G6pc	Glucose-6-Phosphatase
Gapdh	Glyceraldehyde 3 phosphate dehydrogenase
Glut	Glucose Transporter
Gpr	G-Protein coupled receptor
GSH	Glutathione (reduced)
GTP	Guanosin Tri Phosphate
GVDH	Graft Versus Host disease
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HCl	Hydrochloric Acid
HDAC	Histone Deacetylase
HeLa	Henriette Lacks Cells
HEV	High endothelial venules
Hif1α	Hypoxia inducible factor 1α
Hk	Hexokinase
ICAM1	Intercellular adhesion molecule 1
Idh	Isocitrate dehydrogenase
IDO	Indolamine-2,3-dioxygenase
IFNγ	Interferon gamma
IL	Interleukin
IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine based activation motif
Itk	Inducible T cell kinase
IκB	Inhibitor of NF-κB
Lck	Lymphocyte protein tyrosine kinase
LDH	Lactate dehydrogenase
LDHB	LDH subunit B
LFA1	Leukocyte function associated antigen 1
LKB1	Liver Kinase B1
LPS	Lipopolysaccharide

Mapk	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
Mct	Short Monocarbocylate transporter
MHC	Major Histocompatibility complex
NaCl	Sodium chloride
NAD+(H)	Nicotinamide dinucleotide oxidized (reduced)
NADP+(H)	Nicotinamide dinucleotide phosphate (reduced)
NBDG	N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino-2-Deoxyglucose
Ndrg3	N-Myc downstream-regulated gene 3
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor κB
Nrf2	nuclear factor erythroid 2–related factor 2
OA	Osteoarthritis
OCR	Oxygen consumption rate
Oxphos	Oxidative phosphorylation
PAMP	Pathogen associated molecular pattern
PAR	Poly-(ADP) Ribose
Parp1	Poly-(ADP) Ribose polymerase 1
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PCA	Perchloric Acid
Pck1/2	Pyruvate Carboxykinase 1/2
PD1	Programmed Cell death 1
PDH	Pyruvate Dehydrogenase
PDHs	Prolyl-Hydroxylase containing enzymes
PET	Position emission tomography
Pfk	Phosphofructokinase
Pfkfb3	Phsophofructokinase / Fructose-Bisphosphatase
PGC1α	PPARγ coactivator
Pgk	Phosphoglycerate kinase
PI3K	Phosphoinositide 3 Kinase
Pk	Pyruvate kinase
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PNAD	Peripheral node addressin
Pparγ	Peroxisome proliferation activator γ
PPP	Pentose Phosphate Pathway
PRR	Pattern recognition receptors
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RA	Rheumatoid Arthritis

RIPA	Radio immunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rplp0	60S acidic ribosomal protein
S1P(R)	Sphingosine-1-phosphate (receptor)
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS – Polyacrylamide gel electrophoresis
Sgk1	Serum / glucocorticoid kinase 1
shRNA	Short hairpin RNA
Sirt1	Sirtuin 1
Slc	Solute Carrier
SLO	Secondary lymphoid organ
SSC	Side scatter
TAM	Tumor associated macrophages
TBST	Tween containing tris buffered saline
TCA	Tri cyclic acid cylce
TCR	T Cell Receptor
Th	Helper T cell
TLR	Toll like receptor
TNFα	Tumor Necrosis Factor α
Trap1 / Hsp90	Tnf receptor associated protein 1 / Heat shock protein 90
TREC	TCR rearrangement excision circles
Uqcrfs1	Ubiquinol-Cytochrome C Reductase
UT	Untreated
Vegf	Vascular endothelial growth factor
ZAP70	Z-chain associated protein kinase

2 INTRODUCTION

2.1 BASIC ASPECTS OF T CELL MEDIATED IMMUNITY

2.1.1 The constituents of the immune system

The immune system consists of a highly complex network of specialized cells working together to prevent infiltrating viruses and microbes from spreading and thus protecting the body against external pathogens. It can be divided into two main arms, namely the innate and the adaptive immune systems, both fulfilling specific exclusive functions that complement each other to provide the body with optimal protection. Innate immunity delivers broad, non-specific and immediate defense against invading microbes, yet the timeframe of protection is only limited. The adaptive immune response on the other hand is delayed, yet highly specific for the invading pathogen and offers long lasting protection in case of re-infection. Below I will describe in more detail the innate and adaptive defense mechanism of immunity (Murphy, 2011).

2.1.2 The innate immune system

Innate immunity is the evolutionary older arm of the immune system, which developed over 600 million years ago and can be found in 'representative species in almost every level of the evolutionary tree of life' (Cooper and Herrin, 2010). It represents the first line of defense against pathogens acting on several levels that reach from physical barriers to phagocytic engulfment and pathogen clearance by highly specialized phagocytes. Epithelial cells line as physical barriers the entire surface of the human body including the internal lumen of the gut and respiratory tract, generating an isolated environment that is protected from external insults. Beneath this protective barrier tissue resident macrophages and dendritic cells reside that play a pivotal role in host defense and activation of the inflammatory cascade (Murphy, 2011). Their importance becomes clear in the case of tissue disruption such as a wound or in an infection that enables a microbe to overcome the epithelial barrier and enter the body.

2.1.2.1 Pattern Recognition

Immune detection of pathogens is achieved via specialized surface receptors on immune cells called pattern recognition receptors (PRR) that detect microbial

pathogen associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000). It was this groundbreaking finding of PRRs that explained the basic functionality of innate immune detection and the striking difference to adaptive immune activation. The mammalian homologs of drosophila Toll-like receptors (TLR) detect specialized patterns and structures on the surface of microbes. Next to TLR4 that recognizes the gram-negative Lipopolysaccharide (LPS), which was one of the first TLRs discovered, several others have been described, each of them specialized for a certain structure (O'Neill et al., 2013).

2.1.2.2 Inflammation

Inflammation describes the tissue infiltration by activated immune cells that can be initiated by either the detection of an intruding microbe or tissue injury (Medzhitov, 2008). Tissue resident macrophages respond to stimuli such as lipopolysaccharide (LPS) with activation and subsequent release of pro-inflammatory signals including cytokines and chemokines (Haschemi et al., 2012). These soluble inflammatory mediators in turn attract other immune cells and regulate the inflammatory response in order to clear the infection or restore tissue homeostasis (Ahsley, 2012). According to (Medzhitov, 2008) inflammation can be triggered by several stimuli and depending on the type of trigger there will be different physiological and pathological consequences (Figure I).

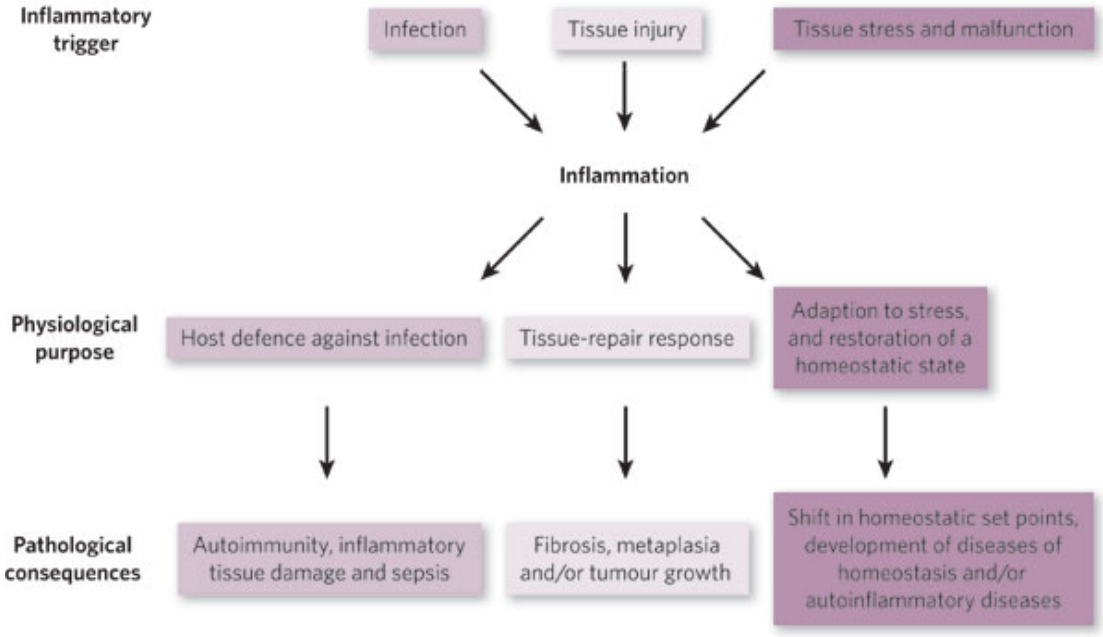


Figure I Origins of chronic inflammation (Medzhitov, 2008)

Different physiological or pathological triggers can cause inflammation that have a specific physiologic purpose and can lead to the development of diseases (pathological consequences).

In acute inflammation, pathogen clearance and tissue repair lead to the resolution of inflammation and the reestablishment of homeostasis that is mediated by several factors in the inflammatory site (Ortega-Gomez et al., 2013). Failure to restore tissue homeostasis leads to the perpetuation of inflammation to a chronic state that contributes to the establishment of chronic inflammatory and autoimmune diseases such as atherosclerosis, rheumatoid arthritis or cancer. This issue will be discussed in a later chapter.

The initiation of the acute inflammatory cascade activates and attracts several innate immune cells such as neutrophils, eosinophils or basophils and monocytes, which differentiate into macrophages once inside the tissue. These granulocytes and phagocytes respectively efficiently clear microbial infections with the release of reactive oxygen species, cytolytic granules and the engulfment of cellular debris, called phagocytosis. As soon as the infection is cleared, anti-inflammatory and inflammation resolving factors, such as Annexin A1 (AnxA1), are released that lead to the resolution of ongoing inflammation and to the reestablishment of homeostasis (Corminboeuf and Leroy, 2014). Although the innate immune system is a firm clearer of microbial infections, it delivers only broad, unspecific and short lasting defense. In order to provide the body with immunity against reoccurring infections a more specific and effective type of immune response has evolved – adaptive immunity.

2.1.3 The adaptive immune system

The adaptive immune system is a specialized arm of immunity that protects the body efficiently from reoccurring infections in an antigen-specific manner. In contrast to the innate part of the immune system, adaptive immune cells comprise only two types, namely B and T cells. For B and T cells to get activated and fulfill their effector functions antigen presentation has to occur (Guermonprez et al., 2002). As the primary focus of this report and work is on T cells, only antigen presentation to this cell type will be discussed below.

2.1.3.1 Antigen encounter and presentation

Primary antigen encounter happens in the periphery where highly specialized antigen presenting cells (APCs) pick up an antigen and start processing it for presentation to the lymphocytes. Only a few professional APCs have been identified so far, including dendritic cells (DC), macrophages and B cells (Guermonprez et al., 2002). Although even T cells have been implicated to function as APCs (Pichler and Wyss-Coray, 1994), only DCs are broadly recognized as the main antigen presenting cell type in the body.

DCs are found in unique locations throughout the body allowing them to efficiently acquire antigen. Positioned on the body's surface areas, such as the skin, pharynx, upper oesphagus, vagina, ectocervix and anus as well as in mucosal surfaces including the gastrointestinal and respiratory tract, DCs inhabit the areas where they are most likely to come in contact with antigen (Steinman and Banchereau, 2007). Once the DC 'senses' the presence of pathogen material, it uses surface scavenger receptors to bind and internalize the antigen. Many scavenger receptors are lectins with a carbohydrate recognition element that enables them to recognize and internalize bacterial- or viral-derived antigens (Steinman and Banchereau, 2007). Other ways of antigen uptake include phagocytosis of microbes or particles and macropinocytosis, which constitute the formation of large pinocytic vesicles containing extracellular fluid and other solutes (Sallusto et al., 1995). These three ways of antigen encounter make the DC such an effective APC and make it possible that pico- and nanomolar concentrations of antigen suffice for efficient antigen presentation (Banchereau and Steinman, 1998).

Once the antigen has been engulfed, it undergoes a complex processing route inside the DC to finally get presented in the form of short peptides bound to Major Histocompatibility Complex (MHC) class I or class II molecules to the lymphocytes in the secondary lymphoid organs (SLO) (Neefjes et al., 2011). Alongside with

antigen processing, the DC starts to mature and migrate to the lymph nodes where it effectively presents the processed antigen to T and B cells and initiate clonal expansion and the onset of the adaptive immune response.

The migration of DCs occurs in a similar manner to lymphocytes, which will be discussed in more detail in a later chapter. Murine DCs are characterized by the expression of CD11c surface marker, yet many different subsets with various surface receptor expression profiles have been described (Merad et al., 2013). Depending on the subsets of DC the way of entry into the LN differs, including DCs entering from the blood (del Hoyo et al., 2002) as well as DCs entering through the afferent lymphatic vessel (Anjuere et al., 1999; Ruedl et al., 2000). The actual antigen presentation to naïve T lymphocytes occurs in the cortex of the LN and requires the physical contact between a DC and the naïve T cell.

(Mempel et al., 2004) could show, using two-photon microscopy, that T cell priming occurs in three distinct phases, consisting of short T cell-DC interactions that result in decrease of T cell motility and upregulation of activation markers. Following, T cells and DCs form stable conjugates lasting for 12 hours and leading to the production of interferon gamma (IFNγ) and interleukin-2 (IL-2). In the last phase T cells regain their high motility characteristics and resume with the initial short interactions with DCs. The third phase of T cell priming also coincides with the onset of T cell proliferation and clonal expansion, characteristic of the adaptive immune response (Mempel et al., 2004). As can be seen, T cell priming depends on physical interaction of antigen-MHC complex on the DC with the T cell receptor (TCR) on the naïve T cell. In order to yield a potent T cell immune response that relies on clonal expansion of the primed T cell, several other signals are necessary to ensure the correctness of the reaction and to avoid inappropriate activation to false signals. The next chapter will describe in more detail T cell activation and the connected proliferative clonal expansion that yields the highly specific antigen dependent immune response.

2.1.3.2 T Cell Activation & Proliferation

The activation of T cells describes the biochemical and morphological events that occur in the naïve T cell after peptide MHC recognition (Smith-Garvin et al., 2009). In recent years the importance of metabolic adaption during immune cell activation has been thoroughly investigated and will be discussed in a later chapter. Below I will describe the downstream signaling cascade that is initiated upon antigen

presentation to the T cell receptor (TCR) and the phenotypical characteristics that change subsequently and lead to T cell activation.

2.1.3.2.1 T Cell Receptor Signaling

Responsible for the recognition of peptide MHC complexes by naïve T cells is the TCR, an integral and defining molecule on the T cell surface. The TCR is a heterodimer consisting of an α- and β-chain that are each anchored to the cell membrane and in complex with the signaling molecule CD3 (Weiss and Stobo, 1984). Although the majority of T cells express the α- and β-chain, in about 5% of T cells these are replaced by a γ- and δ-chain. While the composition of the TCR is an exciting area of research that has important disease implications (Vantourout and Hayday, 2013), it will not be further discussed here.

The TCR signaling cascade involves a series of adaptor and effector molecules that act together to constitute the TCR complex and effectively transduce signals (Figure II) upon interaction with the antigen - MHC complex. The first experimental investigations into TCR signaling could demonstrate a rise in intracellular Ca^{2+} levels in response to phorbol esters and Ca^{2+} ionophores. This release of Ca^{2+} into the cytosol originated from intracellular pools mediated by inositol triphosphate (IP3) that coincided with Ca^{2+} influx from the extracellular space (Imboden et al., 1985). Subsequent studies identified Phospholipase C (PLC) as downstream effector enzyme of the TCR, generating IP3 and Diacylglycerol (DAG) from the hydrolysis of the membrane bound phospholipid inositol 4,5 bisphosphate, which are responsible for Ca^{2+} release (Smith-Garvin et al., 2009).

While these studies have delivered immense contribution to the understanding of the TCR cascade the detailed mechanisms of how the TCR conveyed the signal from the interaction with the antigen-MHC complex remained obscure. It is now clear that one of the earliest events after TCR ligation to the MHC is the phosphorylation of immunoreceptor tyrosine based activation motifs (ITAM) on the cytosolic side of the CD3 ζ-chain mediated by lymphocyte protein tyrosine kinase (Lck). This phosphorylation triggers a chain of events including the recruitment and activation of ζ-chain associated protein kinase (Zap70), which in turn promotes the recruitment of the adaptor proteins Slp-76, Vav, NCK, GADS and inducible T cell kinase (Itk). This complex is then able to phosphorylate the previously mentioned PLC and activate Ca^{2+} signaling.

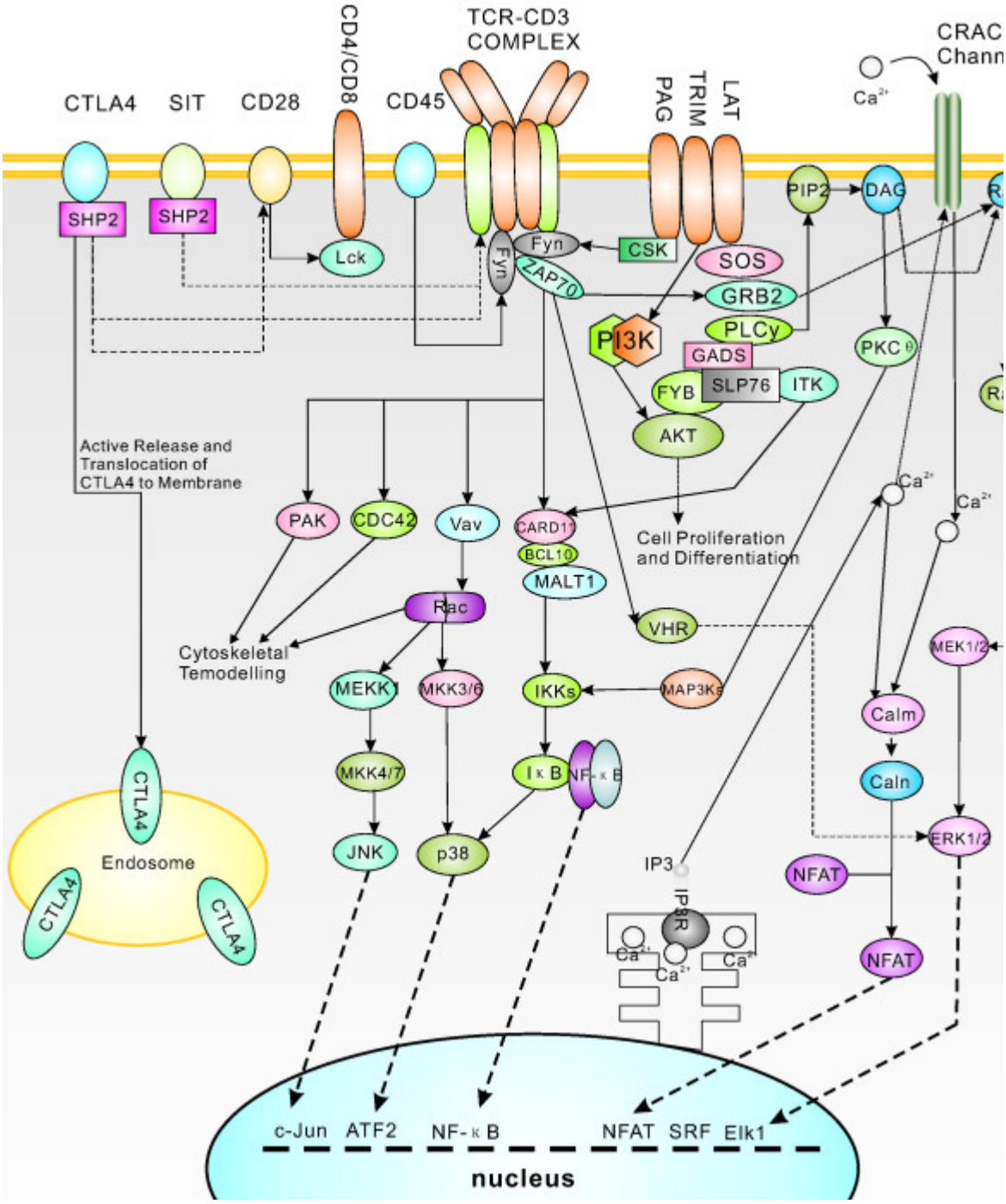


Figure II T cell receptor signaling (Xiang et al., 2010)

Upon antigen recognition, the T cell receptor delays the signal via adaptor molecules and downstream protein and non-protein kinases to transcription factors that in turn activate gene expression and cause physiologic adaption.

The activation of naïve T cells is a complex process that requires not only the TCR signal, but also the stimulation of several other costimulatory receptors (Chen and Flies, 2013). CD28 has been shown to be essential for proper T cell activation as its absence induces T cell anergy (Okkenhaug et al., 2001; Schwartz, 1990) and anergic T cells were found to have defective CD28 signaling (Suzuki et al., 1995).

The triggering of CD28 signaling requires binding of one of its ligands CD80 (B7.1) or CD86 (B7.2) that are expressed on activated DCs. Interestingly initiation of CD28 signaling through either CD80 or CD86 has been shown to be highly redundant as they lead to the same downstream biochemical effects in the activation phase (Acuto and Michel, 2003). The importance of CD28 signaling activation in TCR stimulation has been widely explained by the fact that it lowers the threshold for TCR activation and increases the response duration that in turn leads to stabilized expression of IL-2, which favors T cell survival (Rudd, 1996). The interaction between CD28 and its ligands leads to the phosphorylation of the cytoplasmatic domain of CD28 and subsequent recruitment of PI3K, which has wide reaching effects on T cell fate and function. T cell receptor activation and downstream signaling lead to dramatic changes in gene expression levels, metabolic activity and macromolecule synthesis. This, in turn, enables the T cell to alter its morphology, grow and start to proliferate.

2.1.3.2.2 Phenotypical Changes during Activation

Upon activation of the TCR signaling cascade, the T cell undergoes a plethora of phenotypical and morphological changes that enable it to mount an appropriate adaptive immune response. Naïve T cells are characterized by surface expression of CCR7, L-Selectin (CD62L) and the IL-7R (CD127) that enable them to effectively patrol secondary lymphoid organs and scan for peptide:MHC complexes for their cognate antigen. During the first 12h after antigen recognition the naïve T cells grows in size, starts to produce cytokines including IL-2, IL-4 and IFNγ and acquires an activated surface phenotype by expressing the surface markers CD25, CD44 and CD69. After this initial growth phase, the clonal expansion phase begins in which the T cell starts actively to proliferate (Ley, 2014; Mempel et al., 2004). After successful activation and proliferation, activated T cells start egressing the lymph nodes and travel towards the site of inflammation.

2.1.3.3 T Cell Trafficking

Naïve T cells patrol continuously throughout the lymphatic system and home to SLO's in order to increase the likelihood of successful antigen encounter and initiation of a proper adaptive immune response (Takada and Jameson, 2009). The main route of T lymphocyte entry to SLO's is via High Endothelial Venules (HEV), which represents a highly efficient way of lymph node entry (Mackay et al., 1990; von Andrian and Mempel, 2003). Lymphocyte trafficking and entry into SLO's or target tissue generally involves four different subsequent steps, including tethering,

rolling, activation and arrest (Marelli-Berg et al., 2008). Naïve lymphocytes first initiate tethering to endothelial cells via an interaction between leukocyte receptor L-selectin (CD62L) and its endothelial ligand PNAd – an O-linked carbohydrate moiety, expressed on HEV's. This interaction leads to subsequent rolling on the endothelial surface, mediated by L-selectin and PNAd interactions. Subsequently, firm arrest of T cell rolling is facilitated by the integrin Leukocyte Function-associated Antigen 1 (LFA-1) that interacts with endothelial inter-cellular adhesion molecule 1 (ICAM 1), which allows for transmigration into the lymph node (Figure III) (von Andrian and Mempel, 2003).

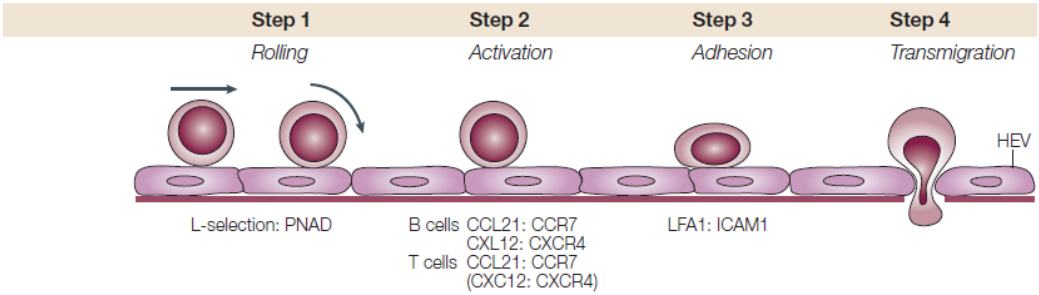


Figure III Lymphocyte tethering, rolling and arrest on HEVs (von Andrian and Mempel, 2003))

Leukocytes enter the SLO's or target tissue via a four-step cascade including tethering to and rolling on the endothelial surface, firm adhesion and finally transmigration through the endothelial layer.

Efficient leukocyte arrest requires the activation of integrins (i.e. LFA1). This occurs via the chemokine receptor CCR7 binding to its ligands CCL19 or CCL21, which are expressed on the surface of HEVs (Marelli-Berg et al., 2008). Although LFA1 can bind both ligands, the relative contribution of CCL19 and CCL21 to leukocyte arrest remains to be determined. Both chemokines are also highly expressed in the T cell zone of the paracortex in the lymph node, in which they increase T cell motility and are believed to maximize antigen-scanning on DCs (Masopust and Schenkel, 2013). It has been estimated that one DC is 'scanned' by up to 500 T cells per hour (Bousso and Robey, 2003).

T cell activation induces the up-regulation of several activation markers, including the hyaluronic acid receptor CD44, a glycoprotein that has been described to define an effector memory phenotype (Yamaki et al., 2014). Interestingly, CD44 deficient mice show normal embryonic and lymphocyte development, yet the homing patterns including the entry into the thymus especially are defective (Protin et al., 1999). Other activation markers include CD25, the alpha chain of the IL-2 receptor and CD69, a C-type lectin protein involved in T cell proliferation (Lauzurica et al.,

2000), which becomes highly expressed during activation. In parallel with these activation markers, the expression pattern of chemokine receptors changes during activation. In contrast to naïve T cells that express CCR7 and L-selectin, allowing efficient homing and entry into the lymph node, T cell activation leads to increased expression of receptors that allow T cell access to non-lymphoid tissue, including the integrins LFA-1 and VLA-4 and chemokine receptors such as CCR5 and CXCR3 on the Th1 subset, CCR4 and Crth2 on the type 2 helper subset as well as CCR6 on the Th17 subset (Islam and Luster, 2012).

After antigen encounter and successful activation and proliferation, T cells egress from the lymph node and start to travel to the site of inflammation. This egress is mediated by a sphingosine-1-phosphate (S1P) – S1P receptor (S1PR) signaling cascade that allows the entry of a T cell into a cortical sinus within the lymph node, hereby paving the way for efficient exit through efferent lymphatics (Pappu et al., 2007). Interestingly, it was shown that S1PR acts mainly to overcome the competitive CCR7 signaling on the T cell, which promotes the retention in the lymph node (Pham et al., 2008).

Directed migration into the inflamed tissue is mostly orchestrated by pro-inflammatory chemokines that are mainly secreted by endothelial cells surrounding the inflammatory site (Nourshargh, 2014), exposed to pro-inflammatory cytokines such as TNFα (Harris et al., 2014). Details of chemokine signaling are described in the next section.

2.1.3.4 Chemokine Signaling

On their way throughout the body, T cells are guided by a family of small chemo-attractant lipid or protein molecules called chemokines that initiate migratory chemotactic response upon binding to a chemokine receptor on the cell surface (Ward, 2006). Protein chemokines are structurally classified into the four groups C, CC, CXC and CX_3C, due to the number and spacing of their N-terminal cysteine residues (Curnock et al., 2002). Directional, chemokine-mediated T cell trafficking is a complex process that involves ligand redundancy, collaboration and antagonism in addition to the emerging concept of biased receptor signaling in which structurally unrelated chemokines activate different downstream signaling pathways from the same receptor (Groom and Luster, 2011; Zweemer et al., 2014).

The signal transduction cascade is mediated via binding of chemokines to a seven transmembrane receptor, coupled to a G-protein (GPCR) that activates a variety of downstream effector molecules, ultimately leading to cytoskeletal reorganization and cell polarization (Mellado et al., 2001). Binding of the chemokine follows a conformational change in the receptor that causes the dissociation of Gβγ subunit and the Guanosin Triphosphate (GTP) bound $G\alpha_i$ subunit of the G-protein (Figure IV). Gβγ activates Phospholipase C (PLC) that leads to the formation of Diacylglycerol (DAG) and inositol triphosphate (IP3), which in turn causes the release of Ca^{2+} from the endoplasmatic reticulum (ER). DAG and Ca^{2+} then act in concert to activate protein kinase C (PKC) and several downstream effector kinases (Mellado et al., 2001).

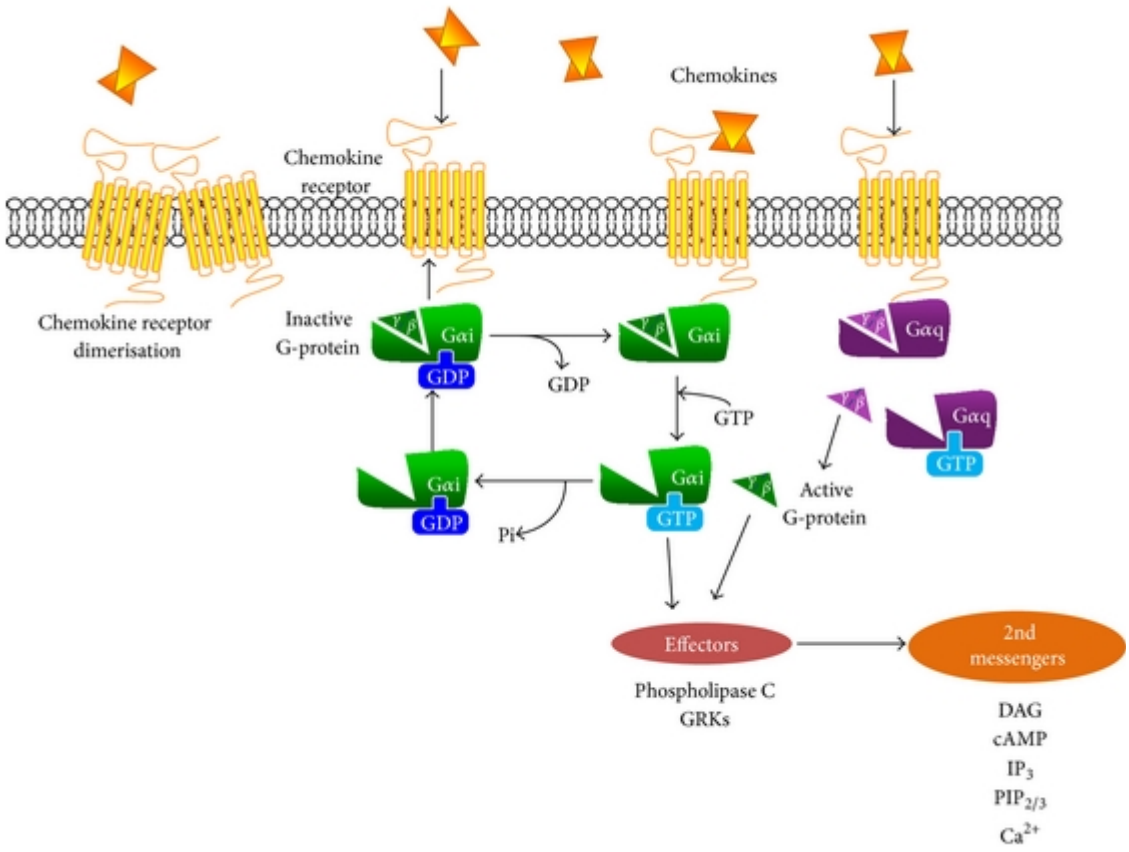


Figure IV Chemokine receptor signaling in T cells (Patel et al., 2013)

The binding of a chemoattractant protein to a chemokine receptor triggers the activation of the attached G protein via dissociation of the βγ subunit from the Gα subunit. Both Gα and βγ activate a variety of downstream effector molecules that lead to the induction of second messenger molecules.

Another class of enzymes that can be activated upon GPCR signaling is the family of phosphoinositide 3 Kinases (PI3K), which represent a central effector molecule in a cell's response to a chemokine (Curnock et al., 2002). PI3K phosphorylates IP3 to

generate the two signaling molecules PIP2 (Phosphatidylinositol 3,4-Bisphosphate) and PIP3 (Phosphatidylinositol 3,4,5-Trisphosphate) that in turn can activate several protein kinases by binding to their pleckstrin homology (PH) domains (Cantley, 2002).

The activation of PI3K is an indispensable event for T cell chemotaxis, yet it seems not to be necessary for directional migration (Cronshaw et al., 2004; Smit et al., 2003). Besides the predominant lipid kinase activity of PI3K, which leads to the phosphorylation of phosphoinositides, PI3K also has direct protein kinase activity and a large array of downstream targets (Thomas et al., 2013). The best-described target of PI3K is the serine / threonine kinase Akt (PKB), which has been shown to be activated by a variety of chemokines including CCL5, IL-8 and CXCL12 (Curnock et al., 2002). The protein kinase Akt (PKB) is a major signaling hub that is involved in the response to cytokines, growth factors and intracellular stimuli and regulates a plethora of cellular outcomes including survival, growth, angiogenesis, proliferation, metabolism and non-the last migration (Manning and Cantley, 2007).

Although key features of T cell mediated immunity have been known for some time, the biochemical aspects of T cell function and migration have only recently come into focus. As it turns out, biochemistry plays a fundamental role in every part of T cell activation and effector function, as will be described in the next section.

2.2 THE BIOCHEMISTRY OF T CELL FUNCTION

2.2.1 Metabolic Adaption during T cell Activation

The biochemical basics for energy production and utilization in the cell have been established in the last century, defining the main metabolic machinery and pathways present in the vast majority of cells. The six-carbon sugar glucose is one of the major energy substrates for the cell, providing its energy in form of ATP that is formed in the catabolic process of glycolysis. In the glycolytic pathway, glucose gets stepwise broken down to two molecules of pyruvate, which is then able to either enter the mitochondria, where it acts as a substrate for the TCA cycle and subsequent oxidative phosphorylation (oxphos) that yields highly efficient ATP production or transformed to lactate and secreted (Figure V). Recently, the importance of metabolism in T cell activation and effector function has been rediscovered as aberrant energy metabolism can lead to changes in cellular behavior and in turn immunological metabolic diseases. As the main research interest in the last years has been to delineate the biochemical pathways activated during T cell activation, I will mostly focus on this aspect of T cell biology in homeostatic settings.

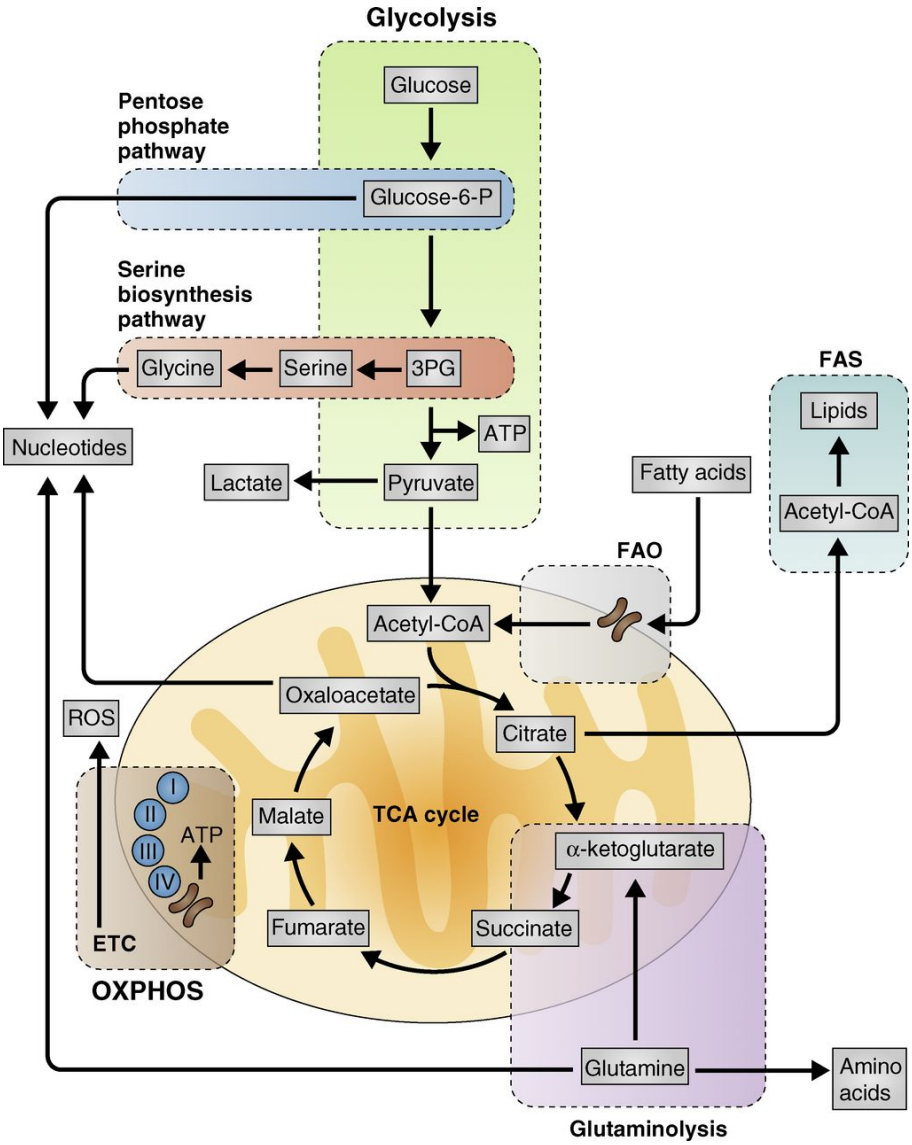


Figure V Main intracellular metabolic pathways (Buck et al., 2015)

The main biochemical pathways that support T cells activation and function include Glycolysis, the TCA cycle and subsequent oxidative phosphorylation in the electron transport chain. Furthermore, fatty acid oxidation (FAO) and synthesis (FAS), amino acid metabolism and the pentose phosphate pathway (PPP) that yields precursors for nucleotide synthesis are critical for T cell activation and function.

2.2.2 T cells increase glycolysis, but mitochondrial oxidative phosphorylation is sufficient for activation

The specificity of an immune response arises from the presentation of antigen to a naïve T cell, which in turn gets activated and starts proliferating at a very high rate, reaching duplication times of only 2 hours (Yoon et al., 2010). In order to achieve such a substantial proliferation capacity, T cells adapt a highly glycolytic metabolism that allows them to provide biosynthetic precursors as well as reducing equivalents in the form of Nicotinamide dinucleotide phosphate (NADPH), required for macromolecule synthesis. Although the engagement of a glycolytic metabolism

in the activation and proliferation of T cells is a well-documented phenomenon (MacIver et al., 2013), it has been recently shown that mitochondrial oxidative phosphorylation (oxphos) is sufficient for the activation of naïve T cells (Sena et al., 2013). In the absence of glucose, but presence of pyruvate and glutamine, $CD4^+$ T cells are still able to up-regulate the activation markers CD25 and CD69 as well as IL-2 mRNA. Pyruvate is an indirect metabolic substrate of oxphos, which is concomitant with the production of ROS due to the leaking of the electron transport chain. Electron leak occurs when the electrons in the respiratory chain exit before the reduction of oxygen to water at the cytochrome c oxidase and instead react with oxygen directly to form the oxygen radical superoxide (Jastroch, 2010). Sena, et.al. (2013) showed the critical involvement of mitochondrial ROS production for the antigen dependent activation process. T cells deficient in mitochondrial complex III failed to induce IL-2 mRNA and nuclear translocation of Nuclear Factor of Activated T-cells (NFAT), a determining transcription factor involved in T cell activation and function (Macian, 2005; Sena et al., 2013). Yet, although oxphos is required and sufficient for T cell activation, over-activation or mitochondrial damage causes increased, harmful ROS production that can lead to apoptosis (Hildeman et al., 1999). It was thus proposed that proliferating cells adapt a glycolytic metabolism in order to reduce oxidative damage and sustain activation and proliferation (Brand and Hermfisse, 1997).

2.2.3 T cell activation requires PPP activity

Increase of aerobic glycolysis and associated up-regulation of the pentose phosphate pathway (PPP) provides a two-way system for the prevention of oxidative damage via ROS. The pentose phosphate pathway is the cells' major source of NADPH, which is required for the production of glutathione, the most abundant reactive oxygen scavenger and regulator of the cellular redox state. Increased intracellular reduced glutathione (GSH) has been associated with enhanced survival rate of activated T cells *in vitro* upon IL-2 withdrawal (Hyde et al., 1997). In Jurkat T cells, the glutamine-induced rise in glutathione levels was also able to protect the cells from apoptosis via induction of IL-2 (Chang et al., 2002).

2.2.4 Amino Acid metabolism supports activation

Glutamine uptake and metabolism has been shown to be crucial for T cell activation and proliferation (Carr et al., 2010). Upon activation glutamine transporters are expressed at the plasma membrane in a CD28-dependent manner and enzyme activities involved in glutamine metabolism in the TCA cycle are enhanced. Accordingly, withdrawal of glutamine from the culture medium in the course of T cell activation leads to decreased proliferation and cytokine production, which cannot be rescued by the supplement of biosynthetic precursors of glutamine (Carr et al., 2010). As glutamine is a major building block for fatty acid synthesis in oxygen-deplete conditions and a direct substrate for the preservation of the TCA cycle, the need of glutamine for T cell activation is not surprising (Fendt et al., 2013).

Glutamine is not the only amino acid that is required for functional T cell activation. The sulfur-containing cysteine – a part of the previously mentioned tri-peptide glutathione – is absolutely necessary for T cells to proliferate (Levring et al., 2012). The authors of this publication showed that early activation events in the T cell are cysteine independent, but as the cells started to proliferate, cysteine transporters were up-regulated and cysteine uptake increased. An earlier study showed that antigen presenting cells release reduced cysteine into the extracellular space to allow for T cell proliferation (Angelini et al., 2002; Edinger and Thompson, 2002). The uptake of cysteine possibly enables the T cell to maintain a reduced intracellular environment promoting macromolecular synthesis for duplication events.

The degradation of tryptophan by macrophages has also been proposed as underlying mechanism leading to decreased T cell proliferation, a mechanism proposed to be important for the induction of tolerance. Macrophages that differentiate in the presence of Macrophage colony stimulating factor (M-CSF) start to express indoleamine 2,3-dioxygenase (IDO), which is responsible for intracellular tryptophan degradation (Edinger and Thompson, 2002; Munn et al., 1999), thereby diminishing extracellular availability of tryptophan. The uptake and metabolism of extracellular amino acids seems to be crucial for T cell activation and clonal expansion. Sinclair et. al. (2013) showed that a number of amino acid transporters were up-regulated during activation and clonal expansion. Large neutral amino acid transporter (Slc7a5)-knockout mice were unable to undergo metabolic adaption upon antigen stimulation and furthermore failed to induce T cell proliferation or effector functions (Sinclair et al., 2013).

2.2.5 Post Translational modifications during T cell activation and function

A variety of post-translational modifications (PTMs) for proteins are known, amongst which are phosphorylation, acetylation, methylation or parylation. As all of them can have immense impact on protein and cellular function, PTMs are tightly regulated and able to change rapidly. Chylek et al. (2014), could demonstrate global changes in the phosphoproteome that occur seconds after T cell receptor activation (Chylek et al. 2014). Similarly, a recent report identified dynamic changes in arginine methylation during the activation of primary CD4+ T cells (Geoghegan et al., 2015). The authors used a novel method of isomethionine methyl-SILAC coupled to arginine methylated peptide enrichment to identify more than 2500 methylation sites on arginine residues that drastically change during the course of T cell activation.

The attachment of an acetyl- group to a protein moiety (acetylation) however, represents the largest group of PTMs investigated to date. From its discovery 51 years ago, protein acetylation has been recognized as one of major regulators of cellular fate and function and is known to be an indicator of the metabolic state of a cell (Verdin and Ott 2015). The TCA cycle intermediate Acetyl-CoA forms the basic molecule that is attached to a lysine residue via histone acetyl-transferases and detached from deacetylases. The latter class of enzymes includes the NAD^+ dependent sirtuins, which are regulated by a wide variety of metabolic signals and play important roles in mitochondrial metabolism, aging and the effects of calorie restriction (Canto and Auwerx, 2009).

Another class of NAD^+ dependent enzymes involved in the regulation of PTMs is the Poly-(ADP Ribose) Polymerases (PARPs), which add long chains of PAR moieties to proteins. PARylation plays important roles in a number of cellular processes including DNA repair, apoptosis, chromatin modifications or mitosis (Kim et al., 2005). It was recently proposed that PARylation could contribute to cellular architecture, stability and flexibility as it has been shown to be involved in a range of cellular stress adaptions (Leung, 2014), which might include T cell activation and functional responses.

2.2.6 Fatty Acid Oxidation promotes T cell activation in inflammatory conditions

Given the above data, T cell metabolism in activation and proliferation seems to be more complex than previously appreciated. Adapting a Warburg-like metabolism

might still be an important hallmark, yet following antigen recognition T cells simultaneously up-regulate several metabolic pathways, including oxidative phosphorylation, amino acid metabolism, TCA cycle and the pentose phosphate pathway during acute infections. Chronic inflammatory conditions might further complicate this issue. A recent report from Byersdorfer et.al. (2013) showed, that alloreactive T cells utilized Fatty Acid Oxidation (FAO) for the activation process in graft versus host disease (GVDH). The authors observed an increase in palmitate utilization in alloreactive compared to syngeneic T cells, which correlated with enhanced IFNγ production. Both mitochondrial acylcarnitine tranporters CPT1a and CPT2 as well as PPARγ coactivator PGC1α were up-regulated, all required for optimal FAO. This could be observed in multiple models of GVDH. Accordingly, blocking FAO by using the selective CPT1a inhibitor etomoxir, resulted in a marked inhibition of T cell proliferation (Byersdorfer et al., 2013). Contrarily, Wang et.al. (2011) found that activation of T cells within the first 24 hours of antibody mediated TCR triggering involved a metabolic adaption that favored the accumulation of glycolytic, glutaminolytic and pentose phosphate pathway intermediates in addition to higher oxygen consumption. Simultaneously, metabolites of the TCA cycle and FAO were markedly decreased. They furthermore could show that Glutamine but not Glucose deprivation led to an inhibition in cell growth and reduction in the biosynthesis of proteins and lipids. Interestingly, the absence of either glucose or glutamine resulted in a marked decrease of cell proliferation. Consistent with their previous results, small molecule inhibition of glycolysis, PPP or glutaminolysis but not FAO halted cell proliferation (Wang et al., 2011).

According to these data it can be clearly seen that activation and proliferation are two distinct features that constitute different metabolic requirements for a T cell. These involve the differential utilization of energy substrates that goes along with the activity of distinct biochemical pathways. According to the physiological or pathological setting, T cells do not seem to merely rely on one specific metabolic pathway or energy substrate to meet their ‘metabolic demands’, but rather adapt to their microenvironment and available energy sources in order to achieve activation or proliferation (Byersdorfer et al., 2013; Edinger and Thompson, 2002; Sinclair et al., 2013; Wang et al., 2011). In light of this it is becoming clear that metabolism, nutrient availability and factors in the surrounding microenvironment affect the function of a T cell on various levels.

2.3 THE INFLAMMATORY MICROENVIRONMENT

Once activated T cells reach the inflammatory site, they are subjected to a variety of different micro-environmental stimuli likely to impact the ongoing immune response. Low glucose and nutrient concentration, oxygen pressure (hypoxia) and decreased pH are commonly associated with chronic inflammation (Haas et al., 2013) (Figure VI). Additionally, elevated levels of ROS, free fatty acid and cholesterol concentrations as well as cell debris and metabolic end products are often found to be concentrated in sites of an inflammation (Haas et al., 2013).

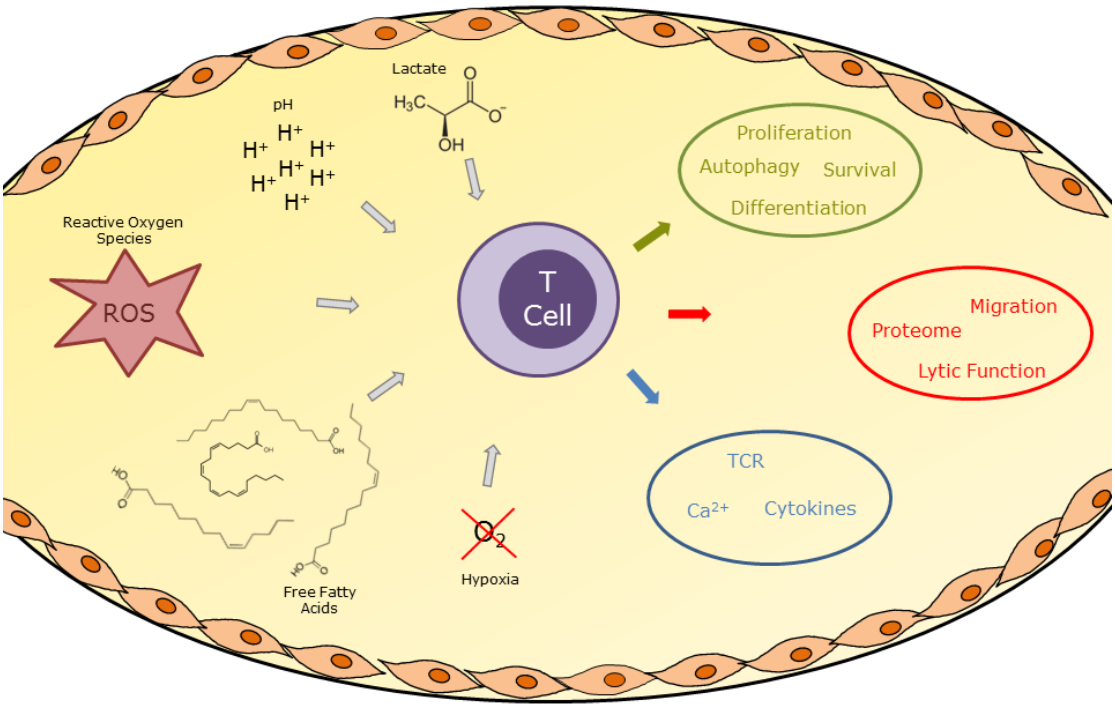


Figure VI Mediators in the Inflammatory Microenvironment affect T Cell Function (Haas et al., 2013)

Soluble factors in the inflamed microenvironment affect T cells in their functional and behavioural patterns. Lactate, low pH, reactive oxygen species, fatty acids of low oxygen concentration exert inhibitory or activating effects on the intruding T cells, controlling the course and outcome of the immune response.

2.3.1 Hypoxia

One aspect of inflamed tissues is, that they are often accompanied by low oxygen concentrations (<2%) also known as hypoxia (Gaber et al., 2013). Not only is oxygen supply in inflammatory sites often reduced due to vascular occlusion and thrombosis (Eltzschig and Carmeliet, 2011), but also infiltrating metabolically active immune cells, resident epithelia and vascular endothelia show high demand for

oxygen (Grenz et al., 2012). This makes it clear that hypoxia and immune cell infiltration are connected in a feed forward loop that promotes the establishment of chronic disease.

The absence of oxygen has profound effects on the function and fate of a T cell by inducing the master transcription factor Hypoxia inducible factor (Hif1α) (Bollinger et al., 2014; Pollizzi and Powell, 2014). Under normoxic conditions (2-3% oxygen) Hif1-α is hydroxylated by prolyl-hydroxy-domain containing enzymes (PDHs), which target it for ubiquitinylation-dependent proteosomal degradation. Hypoxia on the other hand inactivates the PDHs and in turn activates the translocation of Hif1α into the nucleus and transcription of its more than 200 target genes (Ong and Hausenloy, 2012). Targets of Hif1α include genes responsible for energy metabolism, cell differentiation, migration and apoptosis (Liu et al., 2012). Upon exposure to hypoxia, T cells enhance their activation of survival pathways, yet simultaneously decrease their cytokine production, which was proposed to be a protective mechanism against tissue destruction (Gaber et al., 2013; Sitkovsky and Lukashev, 2005). Surprisingly, $CD8^+$ T cells have been shown to be unaffected or even have enhanced cytolytic properties in hypoxic conditions, contrary to their inhibited cytolytic function described in inflammatory conditions (Caldwell et al., 2001).

2.3.2 Reactive Oxygen Species

The presence of reactive oxygen species (ROS) has historically been associated with a decrease in cellular function. Yet some studies show a crucial role for ROS in intracellular signaling, apoptosis induction, cell activation and metabolic feedback mechanisms (Sena and Chandel, 2012). Interestingly, the authors of this study propose a model for ROS-dependent cell activity, whereby the concentration of intracellular H_2O_2 critically determines the cellular response. In homeostatic conditions low ROS levels are sufficient to keep the metabolic and cellular activities constant. Upon exposure to stress stimuli, ROS levels rise to meet the metabolic demands for cell activation and function and are required for proper T cell activation (Sena et al., 2013). T cells isolated from $Uqcrsf1^{-/-}$ mice that show reduced mitochondrial ROS where unable to induce NFAT-dependent IL-2 induction necessary for activation. These cells could not undergo antigen-dependent activation *in vivo*, yet their proliferative potential remained unchanged (Sena et al., 2013). This study elegantly demonstrated the crucial role for mitochondrial ROS in T cell activation. In inflammatory conditions ROS levels are raised due to increased

release of H_2O_2 and superoxide by neutrophils and macrophages (Eltzschig and Carmeliet, 2011). Elevated ROS levels exert cell-damaging effects that ultimately lead to senescence and cell death due to their highly reactive nature (Sena and Chandel, 2012). In addition to that, ROS have been implicated to serve as potent intracellular signaling molecules, altering the proteome of activated T cells both in a qualitative and quantitative manner and making them important mediators in chronic inflammation (Griffiths et al., 2011).

2.3.3 pH

Changes in intra- and extracellular pH can have tremendous effects on cell function and viability. A decrease in pH for a few points from the neutral 7.5 potentially inhibits most cellular functions, including cAMP and calcium signaling, DNA and protein synthesis as well as some enzyme activities (Lardner, 2001). Every enzyme has an optimal pH value that is dependent on the amino acid composition and linked secondary structure of the protein. The pH value affects the ionization state of charged acidic (carboxylic) or basic (amino) functional groups due to modification of hydrogen bonds between the amino acid termini. This leads to denaturation or alteration in the secondary structure of the enzyme that has direct consequences on the confirmation and function of the enzyme.

Interestingly, some studies show extracellular decrease in pH connected to an increase in cellular function. Trevani et.al. (1999) showed that a decrease of extracellular pH to 6.5 enhanced the activation of neutrophils, thereby intensifying the immune response (Trevani et al., 1999). The reduced pH value led to a concentration of intracellular calcium levels that promoted the production of hydrogen peroxide and subsequently enhanced the capability of microbial lysis (Trevani et al., 1999). T cells on the other hand have been found to be negatively affected by a pH reduction. Both migratory properties and cytolytic function were impaired in a low pH environment, attributed to possible changes in the protein conformation states as described above (Lardner, 2001; Redegeld et al., 1991).

In conclusion, the inflammatory microenvironment harbors several physical and chemical factors that influence the activity and function of infiltrating immune cells and might contribute to the progression and outcome of chronic inflammatory diseases. Below, I will describe some characteristics of infiltrating T lymphocytes that have been observed in chronic inflammatory conditions.

2.3.4 Effector T Cell Characteristics in Chronic Inflammatory Conditions (Rheumatoid Arthritis)

Chronic inflammatory diseases, such as rheumatoid arthritis (RA) are a major health burden in the United Kingdom, affecting more than 580,000 people in England and Wales (NHS, 2014). Joint inflammation caused by elevated pro-inflammatory cytokine levels and infiltrating immune cells leads to swelling of the joints, cartilage and bone destruction that causes insufferable pain to the patients. The development of biological agents has brought great advances in the treatment of rheumatoid arthritis. These include the monoclonal antibodies infliximab and Etanercept against soluble TNFα (Scheinfeld, 2004) and the IL1β inhibitor ACZ885 (Alten et al., 2008), which inhibit the activity of these two inflammatory cytokines, reduce joint inflammation and cartilage destruction. Despite this substantial progress some underlying disease mechanisms are still incompletely understood.

RA and other chronic inflammatory disorders are accompanied by infiltrating T cells, which represent the predominant cell type in the site of inflammation (Bankhurst et al., 1976). Increasing evidence suggests that besides genetic dispositions, the prevailing microenvironment contributes to the phenotype of T cells observed in inflammatory conditions (Haas et al., 2013). T lymphocytes present in chronic inflammatory diseases show specific phenotypes that might contribute to the disease development and severity.

Germann et al. (1996) have shown that IFNγ producing Th1 $CD4^+$ T cells are present in the rheumatic synovial joint and their number correlates with disease severity. Additionally, treatment with IL-12, a T helper type 1 (Th1)-inducing cytokine, worsens the disease outcome (Germann et al., 1996). Contrarily, a study from (Pene et al., 2008) shows that T cells isolated from the synovial fluid from arthritis patients mainly display a phenotype skewed towards Th17 polarization and supporting this view, the rheumatoid synovia is enriched in the pro-inflammatory cytokines IL-17, IL-22 and TNFα (Al-Saadany, 2015). In contrast to their homeostatic function, $CD4^+$ T cells show proliferative hypo responsiveness to TCR ligation that might stem from prolonged exposure to high levels of pro-inflammatory TNFα. Synovial $CD4^+$ T cells have been furthermore described to display diminished glycolytic activity, attributed to an insufficient ability to upregulate the glycolytic activator Pfkfb3 (Yang et al., 2013). This deficiency was accompanied by reduced ATP generation and enhanced autophagic activity (Yang et al., 2014), rendering the T cells metabolically inert and prone to apoptosis.

Characterization of rheumatoid arthritis patients has also revealed a distinct phenotype of $CD8^+$ T cells present in the synovial fluid. These cells were found to be mainly activated memory T cells ($CD45RA^-$) with high surface expression of CD80, CD86 and PD1 (Cho et al., 2012). The authors furthermore describe a marked reduction in the cytolytic activity of the cytotoxic T lymphocytes, yet increased expression of IL-4. Intriguingly, several of the glycolytic activator and nutrient sensing proteins, including AKT1, AMPK and LKB1 where shown to control the switch from cytotoxic to effector memory T cells, which also hints toward a glycolytic reduction in synovial $CD8^+$ T cells (Finlay and Cantrell, 2011; Mathis and Shoelson, 2011; Spies et al., 2012).

Overall these data describe a distinct T cell phenotype in chronic inflammatory conditions, including increased pro-inflammatory cytokine production, reduced proliferative and cytotoxic activity as well as generally decreased metabolic activity. Partly, these characteristics might stem from genetic conditions and factors that are independent from the site of inflammation and affect the T lymphocytes systemically. TCR rearrangement excision circles (TREC), products of the TCR rearrangement, can be detected in T cells that recently emigrated from the thymus in to the periphery and is a measure for newly generated T cells from the thymus (Koetz et al., 2000). Blood analysis from RA patients showed decreased TREC values compared with healthy controls, pointing towards a systemic altered T cell dynamic in RA (Koetz et al., 2000). In agreement with the above argument, the majority of peripheral blood T cells in RA were found to be $CD4^+CD28^-$ (Pawlik et al., 2003), a phenotype that was recently shown to produce higher levels of TNFα and IFNγ after TCR crosslinking (Pieper et al., 2014). Interestingly, the same study described T cells showing coproduction of IL-17 only when present in the rheumatic synovia. This finding again suggests that factors in the inflammatory microenvironment may affect the normal function of the cells, which in turn contribute to the disease outcome (Pieper et al., 2014).

Below, I will describe some signaling aspects of lactate, another factor that is commonly found enriched in inflammatory conditions.

2.4 LACTATE AS SIGNALLING MOLECULE

Lactate is a ubiquitous molecule, whose presence in the mammalian body has first been observed in muscle tissue at the beginning of the 19th century (Kompanje et al., 2007). Since its discovery, lactate has been intensely studied and numerous functions in regulating metabolic homeostasis have been defined, which includes the well-known Cori cycle, a metabolic cross talk between the liver and the muscle. Here, muscle tissue metabolizes liver derived glucose to lactate, which in turn is shuttled back to the liver and acts as a fuel source for hepatic gluconeogenesis (Cornell et al., 1973). In the brain on the other hand, lactate acts as a metabolic signal and fuel for oxidative metabolism, which builds the basis of the astrocyte - neuron lactate shuttle hypothesis (ANLS) (Pellerin and Magistretti, 1994).

2.4.1 The Astrocyte - Neuron Lactate shuttle hypothesis

In the brain, lactate has first been postulated to serve as a metabolic fuel secreted from astrocytes and taken up by neurons to enforce oxidative metabolism – a hypothesis known as the astrocyte-neuron lactate shuttle (Magistretti et al., 1994). Early indications came from parallel measurements of blood flow changes, glucose usage and oxygen consumption measured by Positron Emission Tomography (PET) ((Fox and Raichle, 1986; Pellerin and Magistretti, 2003). The authors observed an uncoupling of oxygen consumption from glucose utilization that resulted in a transient elevation of extracellular lactate levels. Interestingly, it was coincidentally reported that astrocytes, a type of glial cells, stimulate glucose uptake and lactate secretion in the presence of the neurotransmitter glutamate (Pellerin and Magistretti, 1994), which was further supported by the observation that glial cells constitute the majority of glucose-consuming cells in the brain (Pellerin and Magistretti, 2003). However, this glutamate dependent glycolytic increase has been challenged in later reports that show how culture conditions, specifically oxygen and glucose concentration in the culture medium dictates the preferred metabolic pathway utilization in astrocytes (Hertz, 2004).

Regardless of whether astrocytes increase their glucose consumption and lactate secretion due to glutamate uptake or not, the generally proposed ANLS hypothesis is explained as follows: During neuronal activation, glutamate is released into the synaptic cleft where it acts as a neurotransmitter. Astrocytes take up the glutamate, which causes an increase in glycolysis with concomitant production and release of lactate. Shuttling lactate back into the extracellular space via short monocarboxylate transporters (Mct) increases lactate availability to neurons and

enables them to use it as fuel for oxidative metabolism ((Magistretti et al., 1994; Tarczyluk et al., 2013). Although there still exist discrepancies of results and inconsistencies in the exact mechanism of the ANLS, the role of lactate as an intra- and extracellular signal still remains.

2.4.2 Lactate Transport

Lactate transport through the plasma membrane is mediated by six so far described short monocarboxylate transporters (Slcs), which are either proton (Mct 1-4) or sodium (Slc5a8, Slc5a12) dependent (Halestrap, 2012; Srinivas et al., 2005). Slc5a8 and Slc5a12 are mainly expressed in kidney and intestine respectively, where they are thought to act mostly as means of lactate reabsorption and dietary short monocarboxylate uptake (Srinivas et al., 2005).

Based on the structural similarity of conserved sequence motifs, the Mct family consists of fourteen members; yet they differ in their substrate specificity, transport kinetics and tissue expression (Halestrap, 2012). Although all transporters of the Mct family are able to transport structurally related short molecules, Mct1 and Mct4 have the highest specificity for lactate transport and show broad tissue expression. Loss of Mct1 has detrimental effects on survival and metabolic homeostasis, as $Mct1^{-/-}$ mice are embryonically lethal and heterozygous $Mct1^{+/-}$ - although developing normal – are resistant to diet induced obesity, underscoring the importance of lactate signaling (Lengacher 2013). Interestingly, Mct1 has not only been shown to locate to the plasma membrane, but also to the mitochondrial outer membrane, where it might facilitate lactate uptake. This supports the hypothesis of that lactate can act as a substrate for oxidation in the mitochondria ((Magistretti et al., 1994; Tarczyluk et al., 2013).

2.4.3 Intracellular lactate signaling pathways

Further evidence that establishes lactate as a signaling molecule comes from the identification of the lactate receptor Gpr81, first cloned in 2001 (Lee et al., 2001). Seven years later, lactate was identified as the primary ligand for Gpr81, being involved in lactate-mediated reduction of lipolysis in adipocytes, the primary Gpr81 expressing cell type (Cai et al., 2008). It is now clear that Gpr81 is a G-protein coupled receptor inhibiting the adenylyl cyclase via the Gi signaling pathway (Ge et al., 2008) and mediates the insulin-induced reduction of lipolysis (Ahmed et al., 2010; Liu et al., 2009). Interestingly, several reports identify the lactate receptor as critical survival signal for cancer cells (Roland et al., 2014; Staubert et al.,

2015), and define it as a therapeutic target in ischemic brain injury (Shen et al., 2015).

Lactate has two primary means of relaying signals into the cell, receptor- and transporter mediated, with the Mct family representing the most abundant lactate transporters (Halestrap and Price, 1999). Once in the cytoplasm, lactate is readily oxidized to pyruvate by the LDH. This reaction proceeds with a concomitant proton transfer from lactate to NAD^+ thereby generating NADH and affecting the redox state of the cell. Although LDH is mainly considered a cytoplasmic enzyme, after years of controversy, the existence of a mitochondrial LDH has finally been proven (Brooks et al., 1999) and in combination with the presence of Mct1 in the mitochondrial membrane (Hashimoto et al., 2006), lactate metabolism is now being considered as an active part of mitochondrial metabolism. Very recently, also the localization of the LDH subunit B (LDHB) to the peroxisomes has been shown in fibroblasts and HeLa cells (Schueren et al., 2014). However, the significance of this finding has not yet been fully elucidated.

Besides the changes in redox signaling, lactate has been shown to affect cellular metabolism in several ways. A report from Leite et al. (2011), shows that lactate inhibits the enzymatic activity of purified phosphofructokinase (Pfk) from murine skeletal muscle, liver and kidney tissues. This effect was mediated by the inhibition of tyrosine phosphorylation on Pfk, and important signal for kinase activation. The authors proposed that lactate might play a role in the inhibition of glycolytic metabolism, hereby serving as an inhibitory feedback mechanism.

A recent report sheds light on another aspect of intracellular lactate signaling as it describes the direct binding of lactate to N-myc downstream-regulated gene 3 (NDRG3) during hypoxia, which is in turn stabilized and executes an ERK1/2-mediated pro-angiogenic program (Lee et al., 2015). This effect is completely independent of Hif1α involvement, which is surprising, as lactate is also known to induce a state of 'quasi-hypoxia' and the activation of Hif1α in various tissues (Colegio et al., 2014a, b; Sonveaux et al., 2012).

Additionally, lactate has very recently been shown to induce the reactive oxygen species superoxide and H_2O_2 in isolated rat hearts (Gabriel-Costa et al., 2015). This study further reports the increase of NADH oxidase and superoxide dismutase 2 activities, which are both indicators of an antioxidant response, as NADH is the main substrate for mitochondrial ROS production. In line with these findings, also Nrf2 and Pgc1α levels were increased, both transcription factors that regulate

mitochondrial biogenesis and improve mitochondrial function (Dinkova-Kostova and Abramov, 2015; Scarpulla et al., 2012).

Thus, it appears that the effects of lactate vary with the type of tissue investigated. So far, only a few reports have addressed the influence of extracellular lactate on the function of immune cells, which I will discuss in the next section.

2.4.4 The effect of lactate on immune cells

Recent reports suggest that lactate acts as an active metabolic signaling molecule in a number of pathologies, which has immense implications for chronic diseases. Studies from (Su et al., 2014) and (Colegio et al., 2014a) show a lactate-mediated effect on macrophages in the tumor microenvironment. Similar to chronic inflammatory conditions, the tumor microenvironment contains high concentrations of lactate, which can reach up to 30mM (Hirschhaeuser et al., 2011). Here, lactate produced by tumor cells is taken up by macrophages where it promotes polarization towards an anti-inflammatory, Arginase 2 (Arg2) expressing M2-like phenotype, via stabilization of Hif1α and increased Vascular Endothelium Growth Factor (VEGF) production. These effects further enhance tumour growth in a malicious loop (Colegio et al., 2014b). Strikingly, Colegio et al. (2014), applied an unbiased high-throughput screen to look for unknown proteins perpetuating this loop, but identified lactate as the orchestrating factor. Similarly, Su and colleagues recently found lactate being the driving force behind tumor associated macrophage (TAM) development during epithelial to mesenchymal transition (Su et al., 2014).

These findings are in line with previous reports that show how targeting the lactate transporter Mct1 in endothelial cells or cervix squamous carcinoma cells rescues lactate-induced Hif1α activation and inhibits the consequential angiogenesis (Sonveaux et al., 2012; Sonveaux et al., 2008). In a follow up study, the authors of these studies could demonstrate that the induction of angiogenesis is mediated via the IkB/NF-κB pathway that drives IL8 expression and leads to increased endothelial migration thereby promoting tumor metastasis (Vegran et al., 2011).

Interestingly, it has been long known that inflammatory sites harbor high concentrations of lactate and that this increase in lactate concentrations stems from a deregulation of cellular metabolism. Although lactate has mainly been considered as a metabolic waste byproduct of glycolytic metabolism, it is now becoming increasingly clear that augmented concentration of lactate can have detrimental

effects in many diseases, most prominently cancer, as it was impressively demonstrated by Su et al. (2014), Colegio et al. (2014) and the group led by Pierre Sonveaux (De Saedeleer et al., 2014; Perez-Escuredo et al., 2015; Van Hee et al., 2015). Despite these observations, the effect of elevated lactate on T cells in chronic inflammatory diseases has not been investigated to date.

3 RATIONALE AND AIM

Acidity is a feature of inflammatory sites such as arthritic synovia, atherosclerotic plaques, and tumor microenvironments and results in part from the accumulation of lactate as a product of glycolysis under hypoxic conditions. It has recently emerged that lactate may be more than just a bystander product of certain metabolic activities / pathways but rather may actively participate to the ongoing immune-inflammatory response.

In light of this, the aim of this project was to elucidate the relative contribution of elevated lactate concentrations on T cell migration and function in the context of chronic inflammation. I hypothesized that lactate in inflammatory sites exerts a feedback mechanism to infiltrating T cells, thereby potentially contributing to some of the T cell characteristics reported in chronic inflammation and subsequently to the establishment and progression of chronic inflammatory diseases (CIDs).

As lactate is a product of glycolysis I was to investigate the metabolic basis of T cell migration and function and to delineate specific biochemical pathways, in particular glycolysis, that define the necessary prerequisites and make it possible for T cells to migrate and function.

4 MATERIALS AND METHODS

4.1 Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Adlrich & Co (UK), unless otherwise specified. A list of antibodies and buffers used can be found in section 8 (Appendix, table 3 and 4).

4.2 T cell isolation, *in vitro* activation and subset enrichment

Lymph nodes isolated from superficial cervical, deep cervical, axillary, brachial, mesenteric, inguinal or lumbar regions, or spleen of C57BL/6 mice (JAX) were smashed through a 45um pore cell strainer to obtain the lymphocytes. The cells were washed and subsequently activated for 3 to 5 days with plate bound 1µg/ml anti-CD3 (clone 17A2, Biolegend) and 5µg/ml anti-CD28 antibodies (clone 37.51, Biolegend), and 10ng/ml IL-2 (PeproTech) in RPMI 1640, supplemented with 10% fetal bovine serum (FBS). $CD4^+$ and $CD8^+$ T cell subsets were isolated with commercially available negative selection magnetic bead isolation kits (Easysep, Invitrogen) according to manufacturer's instructions and experimental settings either prior or post activation. Successful activation of cells was confirmed via light microscopic observation of cell morphology and size. 12h before the assay, the media was changed to RPMI 1640 with 2% FBS.

Human blood was obtained from healthy donors according to ethics approval from Queen Mary Ethics Research Committee (QMERC2014/61). Briefly, Peripheral Blood Mononuclear Cells (PBMCs) were isolated using a single density centrifugation (Histopaque 1077). $CD4^+$ or $CD8^+$ T cells were then isolated from the PBMCs using negative isolation Dynabeads® magnetic separation (Invitrogen), according to manufacturer's instructions. Untouched human $CD4^+$ and $CD8^+$ T cells were stimulated for 5 days with 0.5µg/ml of soluble anti-CD3 (clone Hit3a) and 0.5µg/ml of anti-CD28 (clone CD28.2, Biolegend), together with 10ng/ml IL-2 (Peprotech).

4.3 Chemokinesis assays

Chemokinesis assays were performed in 3 and 5µm transwell inlays (Corning) for naïve and activated T cells respectively. In some experiments, T cells were pre-treated overnight with a number of drugs: 200nM Rapamycin (Calbiochem), 1mM 2-DG, 2mM Metformin (Calbiochem). In most experiments cells were incubated

with 10mM lactic-acid or 10mM sodium lactate 1 hour before the assay and migrated in the presence of lactate, either alone or in combination with 25 µM Phloretin, 425µM α-cyano-4-hydroxycinnamate (CHC), 8 - 92nM AR-C155858 (Tocris Bioscience), 2.5µg/ml Slc5a12 specific antibody (ab107749 Abcam) or 2.5µg/ml Slc16a1 specific antibody (ab90582 Abcam), 25µM PJ-34 or 1µM Ex-527 (Gertz et al., 2013). Briefly, $3x10^5$ lymphocytes were seeded in the upper transwell chamber whereas chemokines (PeproTech) were added to the lower chamber: 300ng/ml CXCL10, 50ng/ml CCL5 or 100ng/ml CCL19/21 of each chemokine. Migrated T cells were counted with a hemocytometer 2, 4 and 6 hours after seeding and percent of migrated cells was calculated.

4.4 Determination of Cell Death

T cell viability upon lactate or metabolic drug treatment was assessed by trypan blue exclusion assay. Whenever necessary, dead cells were removed using Ficoll-Paque Plus (GE Healthcare).

4.5 RNA isolation and reverse transcription

Total RNA was isolated from 10^6 T cells or 10mg RA synovial tissue using RNeasy Mini kit (Qiagen) or Trizol (Life Technologies) according to the manufacturer's instructions and assessed for quality and quantity using absorption ratios of 260/280nm and 260/230nm. Hereby cells were lysed in lysis buffer RLT, nucleic acids were precipitated with 70% ethanol and RNA bound to spin columns. Following several washing steps, RNA was eluted in dH_2O. The isolated RNA was reverse transcribed to complementary DNA (cDNA) using commercially available kits according to the manufacturer's instruction (Applied Biosystems). Briefly, 1ug of total RNA was mixed with buffer, deoxy-nucleotides (dNTPs) and reverse transcriptase and incubated for 2h at 30°C, followed by a 5min heat inactivation step at 85°C. cDNA was diluted to 10ng/µl and stored at -20°C or -80°C for subsequent use.

4.6 qRT-PCR

Quantitative gene expression analysis was performed using SYBR Green Supermix (Biorad) in CFX connect light cycler (Biorad), according to the manufacturer's instructions. Gene relative expression was calculated using the ΔΔct method (Livak and Schmittgen, 2001) and normalized to 60S acidic ribosomal protein p0 (Rplp0).

Primers for qRT-PCR were designed with the assistance of online tools (Primer 3Plus) using at least one exon junction binding-site per primer pair where possible. A complete list of primers used is available in the supporting information (Table S2). Size and specificity of pcr products were confirmed by gel electrophoresis.

4.7 Cell organelle enrichment

Cell compartments were enriched using a Cell Fractionation kit (Abcam) according to the manufacturer's instructions. Here, $6x10^6$ $CD4^+$ T cells were subjected to a series of lysis and centrifugation steps to yield cytosolic, mitochondrial and nuclear fractions.

4.8 Western blot

Total cell lysates were prepared from activated T cells in cell lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) or RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 1% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors (1 mM PMSF plus Complete Mini and PhosSTOP; Roche). Cell lysates were cleared from insoluble material by centrifugation at 14000xg for 10min at 4°C. Protein lysates were boiled at 95°C for 10min in 1x Laemmli buffer (0.1% 2-Mercaptoethanol, 0.0005% Bromophenol blue, 10% Glycerol, 2% SDS, 63 mM Tris-HCl, (pH 6.8)), separated on 4-12% SDS-PAGE and transferred to a Nylon membrane (GE Healthcare) for 2h at 200mA. Membranes were blocked for 2h at room temperature in 5% non-fat dry milk in 0.1% Tween containing Tris buffered saline (TBST) and incubated overnight at 4°C with primary antibodies 1:1000 dilution in 5% non-fat dry milk in TBST. Antibody-antigen complexes were visualized with horseradish peroxidase-conjugated secondary antibody (Amersham Bioscience) (1:5000) against rabbit or mouse and Clarity Western ECL substrate (Biorad). Antibodies against hexokinase 1, pyruvate kinase M1/2, Aldolase A, Enolase 1, Pyruvate Dehydrogenase, Acetyl-Lysine, phospho AMP-Kinase α (Thr172), total AMP-Kinase, phospho acetyl-CoA Carboxylase, total acetyl-CoA Carboxylase, Poly (ADP Ribose) Polymerase 1, Histone H3 and β-actin were purchased from Cell Signaling; antibodies against Slc16a1 and Slc5a12 were purchased from Abcam; Poly (ADP Ribose) (PAR) antibody was purchased from Adipogen (clone 10H). Density of bands of glycolytic enzymes was calculated relative to β-actin via the use of Image J software.

4.9 Enzyme Linked Immunosorbent Assay

Secreted IL-17A was measured in cell culture supernatants from $1x10^6$ $CD4^+$ T cells/ml with a Mouse IL-17A (homodimer) ELISA Ready-SET-Go Assay (ebioscience) according to the instructions.

4.10 Lentivirus preparation and cell transfection

Bacterial glycerol stocks containing sh-RNA plasmid clones targeting Slc5a12 or unspecific RNAs were purchased from Sigma and grown in Luria Bertani broth. Plasmids were isolated using Plasmid Maxi kit (Qiagen). HEK293T cells were grown in 10x10cm cell culture dishes to 70% confluence and transfected with plasmids using the calcium phosphate method. Hereby bis-buffered saline (BBS) and Calcium Chloride ($CaCl_2$) was added drop wise to the diluted plasmid DNA under swirling conditions to form calcium phosphate DNA complexes, which was added to the cells. The supernatant was harvested 48 and 72 hours after transfection, filtered and hundred-fold concentrated in an ultracentrifuge at 20000xg for 2h. Aliquots were stored at -80°C.

4.11 Lentiviral transduction and sh-RNA-mediated gene silencing

Primary $CD4^+$ T cells were isolated and activated as described above. On day 3 of activation, medium was refreshed and cells were incubated with 25µl virus/10^6 cells /ml in the presence of 8µg/ml Hexadimethrine bromide (polybrene, Sigma). The virus was removed 24h later and the T cells were washed twice with PBS and incubated for 24h in complete RPMI culture media. Knockdown efficiency was confirmed by qRT-PCR and Western blot.

4.12 Measurement of extra- and intracellular metabolites and ROS

Lactate concentration was measured in the synovial fluid or peritoneal lavage using the colorimetric lactate assay kit (Biovision) according to the manufacturer's instructions. Intracellular metabolites (NAD^+/NADH, acetyl-CoA, Citrate, Coenzyme A) were measured with colorimetric or fluorescent kits (Biovision) according to the instructions. ATP was measured using a Bioluminescence Assay Kit (Sigma).

Samples were collected after 1x wash in complete RPMI culture media and quenched in liquid nitrogen to stop metabolism. For acetyl-CoA, Citrate, Coenzyme

A and ATP measurements samples were lysed with RIPA buffer and deproteinized with Perchloric acid (PCA) and Sodium hydroxide prior to the assay.

Reactive oxygen species were measured using the fluorescent probe carboxy-H_2DCFDA (Invitrogen, Molecular Probes). Briefly, $CD4^+$ T cells were incubated with 1µM H_2DCFDA/PBS for 30 minutes in the dark. The excess dye was removed with 3x washes in warm PBS and $2.5x10^5$ cells /100µl PBS were plated in a black 96 well plate. After addition of 10mM Sodium Lactate or 1mM H_2O_2 fluorescence was recorded in a time wise manner at Ex 492nm / Em 520nm.

4.13 Glucose uptake, flux, oxidative phosphorylation and fatty acid oxidation

Basal and active glucose metabolism was measured with a Seahorse XF24 or XF96e Extracellular Flux Analyser. Briefly, T cells were grown in high glucose RPMI-1640 supplemented with 10% FCS. One hour before the experiment, $1x10^6$ or $2.5x10^5$ T cells were seeded in a 24 or 96 well micro plate in XF Assay Modified DMEM respectively, in the absence (basal) or presence (active) of 10mM Glucose. CXCL10, sodium lactate, metabolic drugs or PBS were injected during measurement.

Glucose uptake/flux was measured in T cells pre-treated with 1mM 2-DG, 10mM sodium lactate or 10mM lactic acid and then incubated with the fluorescent probes 2-NBDG or 6-NBDG (Life Technologies). Fluorescence was detected at Ex 465nm / Em 540nm.

Rate of basal oxidative phosphorylation was determined by measuring the oxygen consumption rate (OCR) in the presence of glucose. Metabolic stress test analysis was performed by injecting 10mM glucose, 1µM Oligomycin, 1µM FCCP, followed by a combination of 1µM Antimycin A, 1µM Rotenone and 50mM 2-DG. Both assay types were performed using a Seahorse XF24 or XF96e Extracellular Flux Analyser.

Fatty acid oxidation was analysed by measuring the OCR with a Seahorse XF96e Extracellular Flux Analyser according to the manufacturers protocol. Briefly, $2.5x10^5$ T cells were seeded in a 96 well micro plate in XF Assay Modified DMEM containing 2.5mM Glucose and incubated for 45 minutes in a CO_2 free incubator. 15 minutes prior to the assay, control cells were treated with 40µM Etomoxir to block CPT1a. Just before starting the assay, the cells were treated with 167µM BSA-Palmitate or BSA alone. During the assay 1µM Oligomycin and 1µM FCCP were injected.

4.14 Cytotoxic T Lymphocyte differentiation and activity assay

The $CD8^+$ T cell subset were isolated from balb/c mice and incubated with $CD3^+$ depleted and mytomycin C eradicated allogeneic splenocytes from C57BL/6 mice for 5 days. Differentiated Cytotoxic T lymphocytes (CTLs) were enriched with Ficoll-Paque Plus (GE Healthcare) and CD3 enrichment kits and co-cultured with endothelial cells (C57BL/6) in the presence or absence of 10mM lactic acid or sodium lactate. Dead cells were counted using trypan blue exclusion assay 2, 4, 6 and 18 hours after the start of the assay.

4.15 T helper subset differentiation

T cells were isolated from murine lymph nodes and enriched for $CD3^+$ and subsequently $CD4^+$ subsets. 10^6 cells were plated per well and differentiated towards Th0, Th1, Th2 and Th17 phenotype for 3 days with plate bound 1µg/ml anti-CD3 and 5µg/ml anti-CD28 antibodies (BioLegend). Conditions were: Th0 (10ng/ml IL-2); Th1 (10ng/ml IL-2; 3.4ng/ml IL-12; 2µg/ml Anti-IL4); Th2 (10ng/ml IL-2; 10ng/ml IL-4; 2µg/ml Anti-IFNγ); Th17 (10ng/ml IL-6; 2µg/ml Anti-IL-4; 2µg/ml Anti-IFNγ, 5ng/ml TGFβ). All antibodies and cytokines were purchased from PeproTech. Differentiation was confirmed via qRT-PCR, assessing expression of IFNγ for Th1, IL-4, IL-5, IL-13 for Th2 and IL-17 for Th17 cells.

4.16 Intracellular cytokine & transcription factor staining

Murine T cells were incubated in permeabilization/fixation buffer (ebioscience) overnight at 4°C. Samples were washed in permeabilization buffer (ebioscience) and stained for the cytokines IFNγ and IL-17A, using fluorescently conjugated primary antibodies (1:200, ebiosciences) at 4°C for 30 minutes. Human isolated $CD4^+$ T cells were treated as described above. In the final 6h of culture, cells were treated with 50ng/ml phorbol 12-myristate 13-acetate (PMA) and 500ng/ml ionmycin and 3µg/ml Brefeldin A (Sigma-Aldrich), followed by surface staining for CD4 (clone RPA-T4, Biolegend). Cells were fixed and permeabilized as described above, followed by staining for IL-17A (clone BL168, Biolegend) for 30 minutes at room temperature. All intracellular staining was assessed by flow cytometry using a LSR Fortessa (BD Biosciences) and FlowJo version 7.6.5 software.

4.17 Intracellular Immunofluorescence staining and microscopy

Murine CD4 T^+ cells were incubated for 5 minutes with 300nM MitoTracker Deep Red FM (Molecular Probes) at 37°C. Cells were washed in PBS and fixed/permeabilized in permeabilization/fixation buffer (ebioscience) overnight at 4°C. Samples were washed in permeabilization buffer (ebioscience) and incubated with primary antibodies against Hexokinase 1 (1:200, Cell Signaling Technology) for 1h at room temperature. The samples were then incubated with fluorescent secondary antibodies (1:200) for 1h at room temperature and 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes before they were spun down for 5minutes to a cover slip in a cytospin centrifuge. Cells were mounted in Mowiol and pictures taken on a confocal laser scanning microscope LSM510 (Zeiss).

4.18 Human RA synovial tissue collection and immunohistology/ immunofluorescence

RA synovial tissue was collected after informed consent (LREC 07/Q0605/29) from a total of 16 RA patients undergoing total joint replacement or ultrasound-guided synovial biopsies as previously described (Humby et al., 2009). A summary of the demographical and clinical characteristics of the RA patients is reported in Table I. For total T cell scoring, paraffin sections were stained for CD3 and a semi-quantitative score was applied as previously described (Croia et al., 2013). For Slc5a12 single and double immunofluorescence with CD4 or CD8, after antigen retrieval (S2367, Dako) and block of non-specific binding, slides were incubated with primary antibodies either overnight at 4°C (CD4 and CD8, 1:50, Dako) or 1 hour at room temperature (Slc5a12, 1:50, Novus Biologicals) followed by fluorochrome-conjugated secondary antibodies (Invitrogen, Eugene, Oregon, USA). All sections were visualised using a Zeiss fluorescence microscope. Quantification was performed by calculating the percent of double positive $CD4^+$ $Slc5a12^+$ population within the $CD4^+$ or $Slc5a12^+$ cells, or the percent of double positive $CD8^+$ $Slc5a12^+$ population within the $CD8^+$ or $Slc5a12^+$ cells. The number of positive cells, constituting single and double positive for each marker, was counted in 6 images per condition.

4.19 *In vivo* peritoneal recruitment model

All the *in vivo* experiments were conducted under the UK Home Office regulation (PPL 70/7443). Activated T cells ($5x10^6$/mouse) were pre-treated overnight with

200nM Rapamycin, 1mM 2-DG or 2mM Metformin, then labelled with the fluorescent cell dye 7-Hydroxy-9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One (DDAO, Invitrogen) and injected intravenously into syngeneic female C57BL/6 mice that had 3 hours prior received an intra-peritoneal injection of 120ng CXCL10 per mouse. 24 hours after injection, mice were sacrificed and spleen and peritoneal lavage were harvested. T cells were stained for surface markers CD4 and CD8 (ebiosciences) and analysed by flow cytometry. Cells were first gated on CD4 and subsequently analysed for DDAO positivity.

4.20 *In vivo* zymosan-induced peritonitis

C57BL/6 mice were injected with 1mg zymosan per mouse in the peritoneal cavity or left untreated. On day 5, 50µM Phloretin, 5µg/ml anti-Slc5a12 antibody or 5µg/ml anti-rabbit IgG isotype control antibody (Invitrogen) were injected into the peritoneal cavity. 24 hours later, mice were sacrificed and the peritoneal lavage was harvested. T cells were stained for surface markers (CD4 and CD8; ebiosciences) and analyzed by Flow Cytometry. Alternatively C57BL/6 mice were injected in the peritoneal cavity with zymosan (1mg per mouse) on day 0. On day 5, activated $5x10^6$/mouse $CD4^+$ T cells labelled with 3.3µM of the fluorescent cell dye Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) were co-injected with 5µg/ml anti-Slc5a12 antibody, 50µM Phloretin or 5µg/ml of an IgG isotype control antibody in the peritoneal cavity. 24 hours later, mice were sacrificed and the peritoneal lavage and the spleen were harvested. T cells were stained for surface markers (CD4 and CD8; ebiosciences) and analyzed by Flow Cytometry. Cells were first gated on CD4 and subsequently analysed for CFSE positivity.

4.21 Flow Cytometry

Isolated T cells were stained for surface markers; CD3, CD4, CD8, CD25, CXCR3, CCR7, CD62L and LFA-1 with fluorescently conjugated primary antibodies (1:200, ebiosciences) at 4^oC for 30 minutes, and assessed by flow cytometry using a LSR Fortessa (BD Biosciences) and FlowJo version 7.6.5 software.

4.22 Statistical analysis

Data are expressed as mean ± s.e.m. Two-tailed Student's t-test was used to compare 2 groups with parametric data distribution. For multiple comparison analysis, 1-, 2- or 3-way ANOVA was used. In all case, a p-value of less than 5% was considered to be significant.

5 RESULTS

5.1 CHAPTER I – EXTRACELLULAR LACTATE ALTERS MIGRATORY AND FUNCTIONAL PROPERTIES OF T CELLS

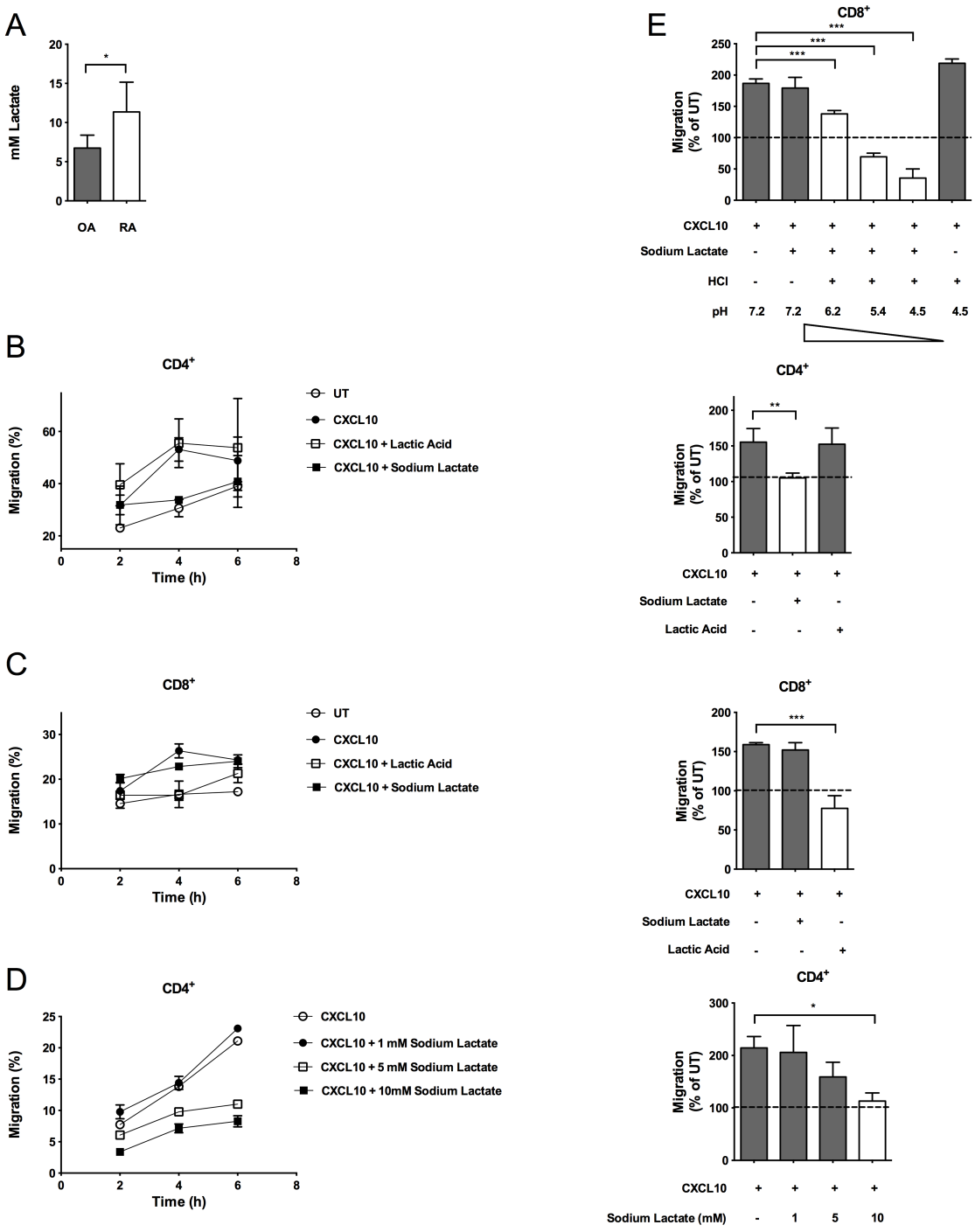
5.1.1 Lactate inhibits chemokinesis of activated T cells

To assess whether T cell motility is affected by lactate, I performed chemokinesis assays of activated T cell subsets in the presence of 10mM lactic acid or sodium lactate. $CD4^+$ and $CD8^+$ T cells were separately isolated using negative selection beads, activated with anti-CD3 and anti-CD28 antibodies and IL-2 for 5 days and the chemotactic response was induced by the pro-inflammatory chemokine CXCL10. The lactate concentrations I used could be measured in the synovial fluid of osteoarthritis (OA) and RA patients (Figure 1A) and have been found in a number of inflammatory sites (Gobelet and Gerster, 1984; Young et al., 2013). I found that $CD4^+$ T cell chemokinesis was inhibited by sodium lactate whereas that of $CD8^+$ T cells was inhibited by lactic acid but not vice versa (Figures 1B, C).

Chemokinesis can be activated by several structurally unrelated chemokines, leading to different responses (Mueller et al., 2007). I therefore also tested the chemokinetic effect of lactate on another inflammatory chemokine CCL5. CCL5-induced motility of $CD4^+$ T cells was again decreased in sodium lactate- but not lactic-acid-rich environment (Figure 1F), suggesting a broader action of lactate in chemokine-induced signaling and downstream effects than previously anticipated. Motility of $CD4^+$ T cells upon sodium lactate treatment decreased with increasing concentration of sodium lactate with an EC50 of about 10mM sodium lactate (Figure 1D). These concentrations of sodium lactate and lactic acid did not affect cellular viability (Figure 1G).

As acidification 'per se' could affect cellular motility (Lardner, 2001), I performed additional chemokinesis assays to discriminate the effects of lactic acid on the motility of $CD8^+$ T cells from the effects of pH reduction in the culture media due to the addition of 10mM lactic acid. Lactic acid is present in solution either as the acid in its associated form at low pH or as the ion salt (i.e. sodium lactate) at higher / neutral pH. I found that progressive acidification of medium containing 10mM sodium lactate reduced the motility of $CD8^+$ T cells (Figure 1E). The stepwise acidification of sodium lactate containing media increases the availability of lactic acid. Importantly, neither the presence of sodium lactate in the culture media alone nor the sole acidification of the media to pH 4.5 with HCl had any effects on the

motility of $CD8^+$ T cells (Figure 1E). These data suggest that inhibition of $CD8^+$ T cell motility by lactic acid cannot be simply ascribed to its effect on the pH reduction in the culture media. Rather the simultaneous availability of lactate and protons (H^+) is necessary to regulate the motility of $CD8^+$ T cells. This effect did not apply to $CD4^+$ T cells since their migration was not affected in lactic acid-enriched medium (Figure 1B).



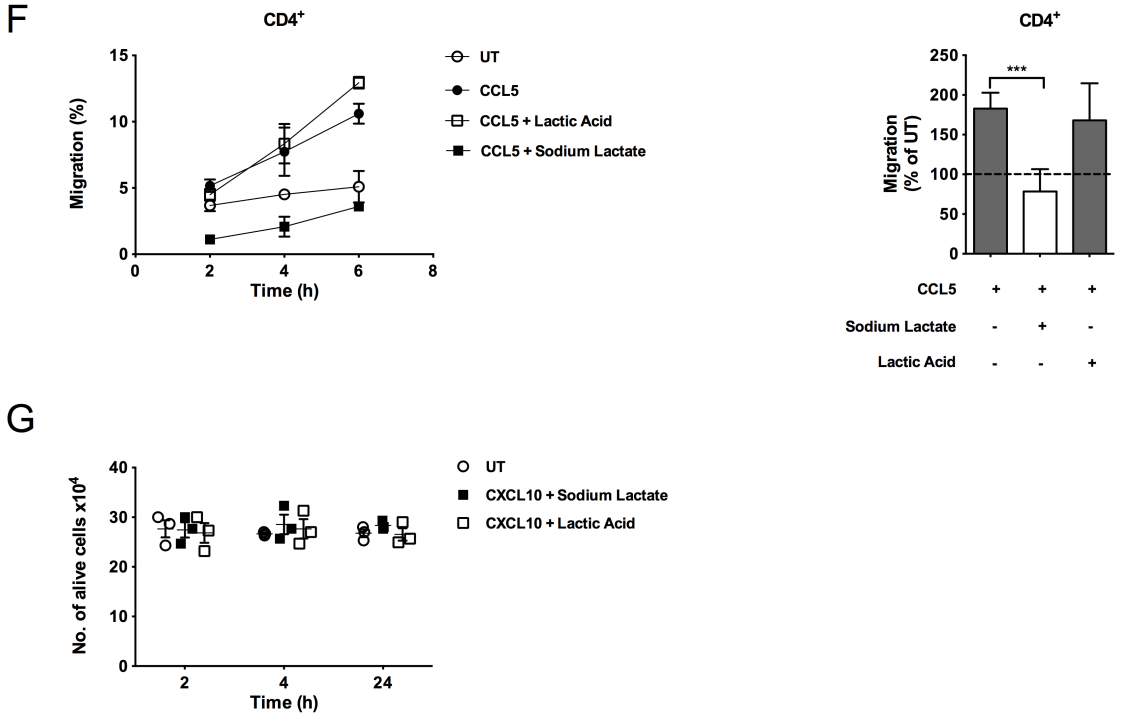


Figure 1 Lactate inhibits effector T cell migration

(**A**) Lactate measurements in the synovial fluid of osteoarthritis (OA) or rheumatoid arthritis (RA) patients. (**B-C**) *In vitro* chemokinesis of activated $CD4^+$ (**B**) and $CD8^+$ (**C**) T cells towards CXCL10 (300ng/ml) in the presence of lactic acid (10mM) or sodium lactate (10mM) shown as kinetic (left panel) and 4h time point (right panel). (**D**) *In vitro* chemokinesis of activated $CD4^+$ T cells towards CXCL10 (300ng/ml) in the presence of increasing concentration of sodium lactate shown as kinetic (left panel) and 4h time point (right panel). (**E**) *In vitro* chemokinesis (4 hour time-point) of activated $CD8^+$ T cells towards CXCL10 (300ng/ml) in the presence of sodium lactate (10mM) or HCl (pH 4.5) alone, or sodium lactate in combination with increasing concentrations of HCl to obtain progressively reduced pH as indicated. (**F**) *In vitro* chemokinesis of activated $CD4^+$ T cells towards CCL5 (50ng/ml) in the presence of lactic acid (10mM) or sodium lactate (10mM), shown as kinetic (left panel) and 4h time point (right panel). (**G**) Total cell number of viable $CD4^+$ T cells treated with CXCL10 in the presence of lactic acid (10mM) or sodium lactate (10mM). (**A-G**) Values denote mean ± SD. (**A**) OA, n=5 and RA n=8 (biological replicates, each measured in triplicate), (**B-G**) Data is representative of three independent experiments; n=3. *P<0.05; **P<0.01; ***P<0.001.

5.1.2 The effects of lactate on different T cell subsets are mediated by distinct transporters

I next investigated the molecular basis of the differential and mutually exclusive responsiveness of $CD4^+$ and $CD8^+$ T cells to sodium lactate or lactic acid, respectively. Fischer et al. described the expression of SLC16A1 / MCT1 by human cytotoxic ($CD8^+$) lymphocytes, a lactate - H^+ symporter which facilitates lactic acid uptake (Fischer et al., 2007). SLC5A12 is the only sodium-coupled lactate transporter described so far (Srinivas et al., 2005). I found that murine $CD8^+$ and $CD4^+$ T cells selectively express SCL16A1 and SCL5A12, respectively (Figure 2A), suggesting a specific functional role of each transporter on the respective T cell subset.

I subsequently sought to confirm that the differently expressed lactate transporters were functionally active in the inhibition of T cell chemokinesis. Blockade of SCL16A1 on $CD8^+$ T cells with the selective small molecule inhibitors phloretin, CHC (Morris and Felmlee, 2008) and AR-C155858 (Ovens et al., 2010), restored chemokinesis of $CD8^+$ T cells exposed to lactic acid (Figures 2B and 2C). The same effect was observed using a specific antibody targeting SCL16A1, yet not with an isotype control antibody (Figure 2B). Conversely, chemokinesis of $CD4^+$ T cells in sodium lactate rich media was recovered following selective reduction of *Slc5a12* expression on $CD4^+$ T cells with lentiviral delivered, specific shRNAs or a specific antibody (Figures 2D and 2E). The anti-SCL5A12 antibody or shRNAs targeting *Slc5a12* did not affect $CD8^+$ T cell migration (Figure 2F). Similarly, the SCL16A1 inhibitors phloretin, CHC and AR-C155858 or the anti- SCL16A1 antibody did not affect the migration of $CD4^+$ T cells (Figure 2G).

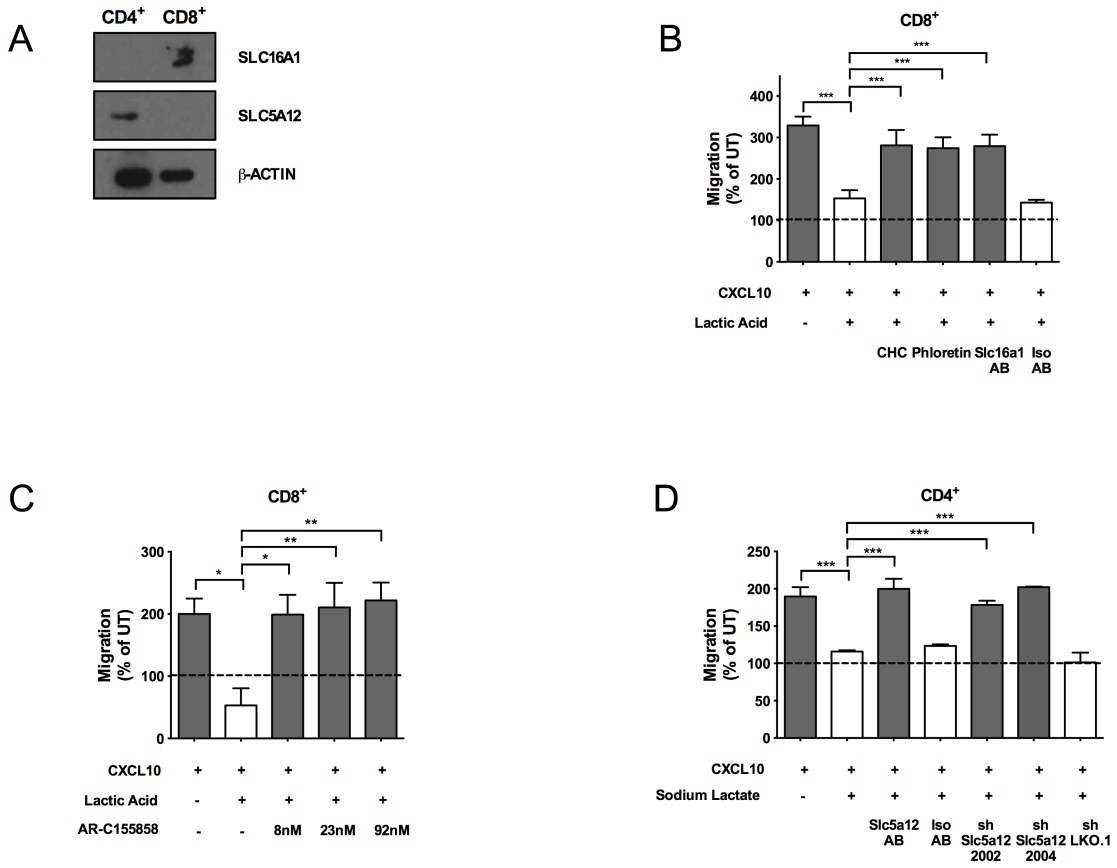


Figure 2 Sodium lactate and lactic acid act selectively on CD4$^+$ and CD8$^+$ T cell subsets through specific cell membrane transporters.

(**A**) Total protein levels of the transporters SCL16A1 and SCL5A12 as assessed by western blot in activated CD4$^+$ and CD8$^+$ T cell subsets. (B–D) *In vitro* chemotaxis (4h time point) of activated CD8$^+$ T cells towards CXCL10 (300ng/ml) in the presence of lactic acid (10 mM) alone, or in combination with CHC (425μM), phloretin (25μM), or anti-Slc16a1 antibody (2.5μg/ml) (**B**), or increasing concentrations of AR-C155858 as indicated in the figure (**C**), and activated CD4$^+$ T cells towards CXCL10 (300ng/ml) in the presence of sodium lactate (10mM) alone, or in combination with an anti-SCL5A12 antibody (2.5μg/ml) or two specific shRNAs (**D**). An isotype control antibody has been included to control for antibody specificity (**B, D**), and a nonspecific shRNA has been included to control for gene knockdown specificity (**D**). (**E**) qRT-PCR and western blots with *Slc5a12*-specific primers and RNAs from activated CD4$^+$ T cells expressing the shRNAs shown.

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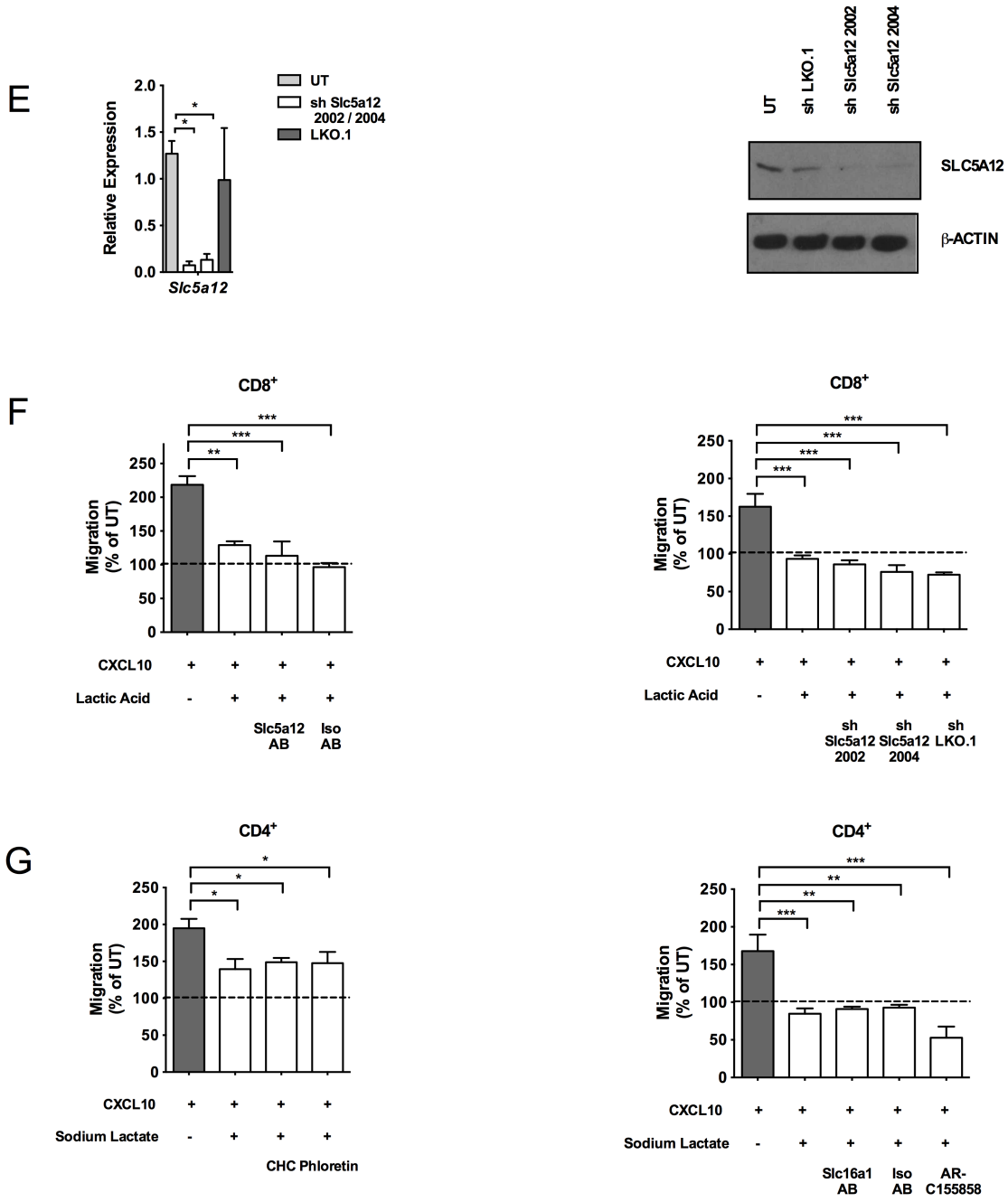


Figure 2 continued

(**F, G**) *In vitro* chemotaxis (4 h time point) of activated CD8$^+$ T cells towards CXCL10 in the presence of lactic acid (10mM) alone or in combination with an anti-SCL5A12 antibody (2.5µg/ml) or an isotype control antibody (B left panel) and two specific shRNAs for *Slc5a12* or a nonspecific shRNA (B right panel), and activated CD4$^+$ T cells towards CXCL10 in the presence of sodium lactate alone or in combination with CHC (425µM) or phloretin (25µM) (**C** left panel) and an anti-SCL16A1 (2.5µg/ml) or an isotype control antibody, or AR-C155858 (8nM) (**C** right panel) (**B–G**). Values denote mean ± SD. Data is representative of three independent experiments; n=3. *P<0.05; **P<0.01; ***P<0.001.

5.1.3 Lactate modulates effector T cell functions

Having established that extracellular lactate inhibits the migration of T cells, I next asked whether cytokine production and effector functions are also affected. I consequently induced polarization of $CD4^+$ T cells towards Th1, Th2 and Th17 subsets in the appropriate cytokine "milieus" (Fitch et al., 2006; Zhu et al., 2010). The expected patterns of cytokine expression by differentiated T helper subsets were confirmed at the mRNA level (Figure 3A). I then tested the effect of the presence of 10mM sodium lactate for 6 hours on the production of cytokines by the different T helper subsets in the same polarizing conditions. Gene expression analysis showed that treatment with sodium lactate caused a significant up-regulation of *Il17* in all the T helper subsets (Figure 3A). Supporting these data, gene expression of *Rorc*, the signature transcription factor of Th17 cells, was also significantly elevated in all the Th subsets upon T cell exposure to sodium lactate (Figure 3A). Gene expression of Th1 and Th2 signature cytokines was either unmodified upon treatment with sodium lactate or even reduced (i.e. *Il4*, *Il5* and *Il13* in the Th17 subset; Figure 3A). Intracellular staining experiments confirmed the increased expression of IL-17 protein in $CD4^+$ T cells exposed to sodium lactate as compared to cells left untreated (Figure 3B). In support of this, pre-incubation with the antibody anti-SLC5A12 reduced the up-regulation of *Il17* and *Rorc* genes induced by sodium lactate, yet not completely abolished it (Figure 3C).

Cytotoxic T cells (CTLs) differentiating from the $CD8^+$ subset express and release cytolytic granules consisting of perforin/granzyme complexes, which promote the killing of target cells (Metkar et al., 2002). To test whether these functions were affected by lactate, I performed cytotoxicity assays with allogeneic endothelial cells. Similarly to the results obtained in migration assays (Figure 1C), lactic acid but not sodium lactate inhibited the cytolytic activity of CTLs and promoted survival of the allogeneic target cells (Figure 3D)

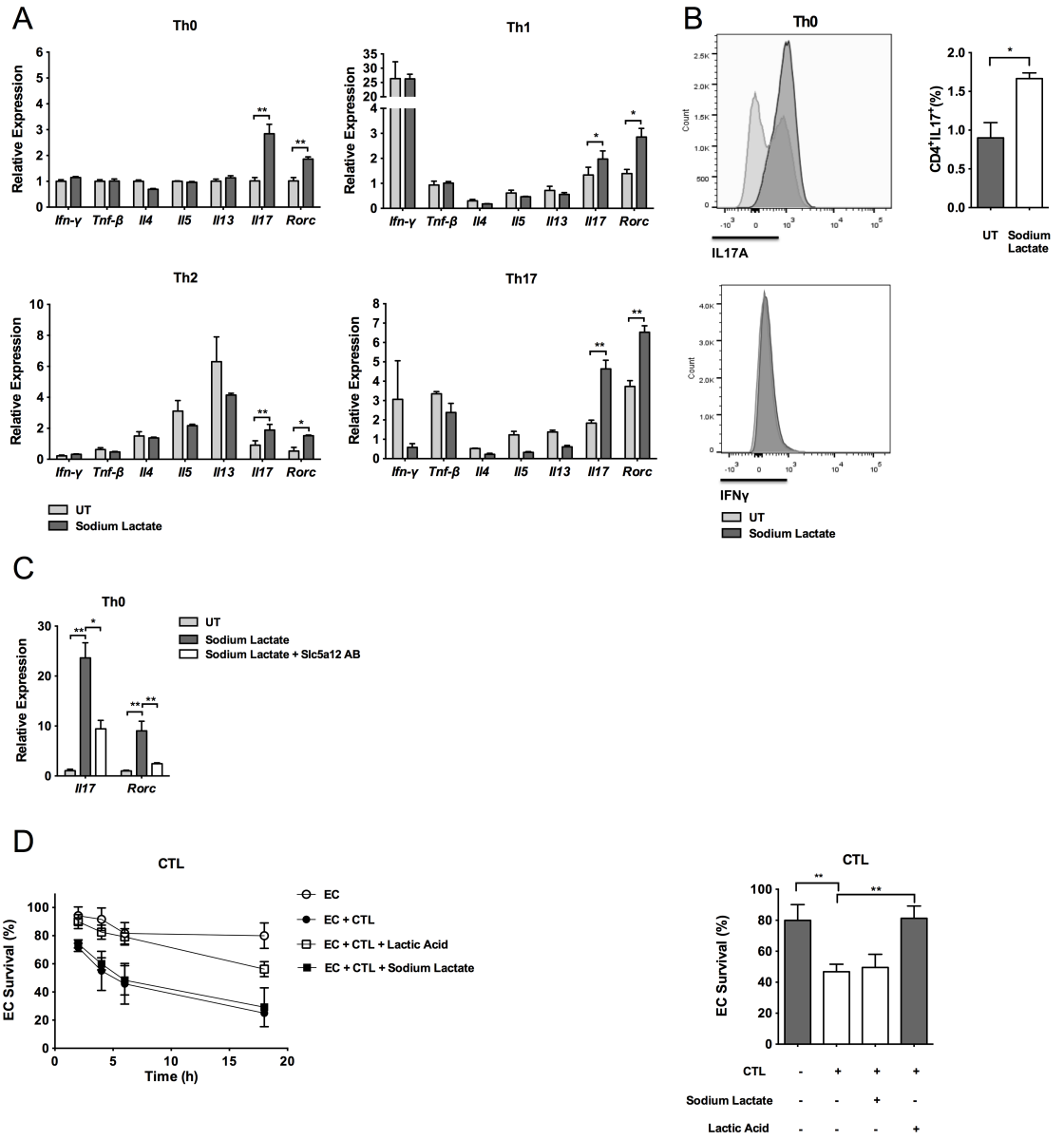


Figure 3 Lactate modulates effector T cell function

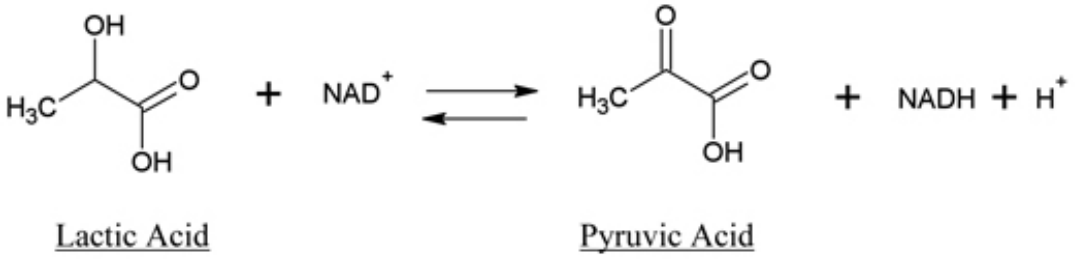
Lactate modulates effector T cell functions. (**A**) Relative mRNA expression levels of the cytokines Ifnγ, *Tnfβ*, *Il4*, *Il5*, *Il13* and *Il17* and of the transcription factor *Rorc* as assessed by qRT-PCR in $CD4^+$ Th subsets Th0, Th1, Th2 and Th17 treated with sodium lactate (10mM) or left untreated. mRNA levels of each cytokine expressed by untreated Th0 cells were set to 1. (**B**) Intracellular staining of IL-17A and IFNγ in activated $CD4^+$ T cells treated with sodium lactate (10mM) or left untreated. (**C**) Relative mRNA expression levels of *Il17* and *Rorc* in activated $CD4^+$ T cells treated with sodium lactate alone or in combination with an anti-SLC5A12 antibody. mRNA levels of untreated T cells were set to 1. (**D**) Cell survival of allogeneic endothelial cells in the presence of $CD8^+$ cytotoxic T cells and lactic acid (10mM) or sodium lactate (10mM) shown as kinetic (left panel) and 6h time point (right panel). (**A-D**) Values denote mean ± SD. Data is representative of three independent experiments; n=3. *P<0.05; **P<0.01; ***P<0.001.

5.2 CHAPTER II – METABOLIC CHANGES INDUCED BY LACTATE

5.2.1 Lactate changes Redox state, acetyl-CoA and Citrate availability

The lactate transporter specificity (Figure 2A) and the T cell insensitivity to lactate upon transporter inhibition (Figures 2B-D and 2F, G) suggest that the effects of lactate are mediated by intracellular signaling, possibly interfering with the cell metabolic machinery. One of the most prominent enzymes that act on lactate inside the cell is considered to be the cytoplasmic lactate dehydrogenase (LDH). The LDH functions as bidirectional enzyme either generating lactate from pyruvate with concomitant oxidation of NADH to NAD^+ at high pyruvate concentrations or oxidizing lactate to pyruvate coupled to a reduction of NAD^+ in the excess of lactate (Veech, 1991)(Equation 1).

Equation 1 Lactate dehydrogenase reaction



To test the involvement of the LDH, I measured the abundance of total NAD^+ and NADH in activated $CD4^+$ T cells exposed to 10mM sodium lactate. As expected, 10 minutes after lactate addition, most of the available NAD^+ was reduced to NADH as can be seen in a drastic drop in NAD^+ levels compared to baseline, with simultaneous increase in NADH levels (Figure 4A). Surprisingly, after 30 minutes and up to 1h of lactate treatment the NADH levels fell back to baseline with only slight increase in NAD^+, pointing towards a part re-oxidation and part biosynthetic use of NADH (Ying, 2008). Although NADH has many biological functions in the cell, a key function of NADH is to act as a cofactor in the NADH dehydrogenase / complex I of the mitochondrial electron transport chain (ETC). The ETC generates ATP and ROS during its activation (Finkel and Holbrook, 2000). In line with a mitochondrial oxidation of NADH, I found that the levels of ROS after 20 and 30 minutes of lactate exposure were increased up to 3.5 and 2 fold respectively (Figure 4B). Interestingly, ROS levels transiently increased already after 5 minute exposure to lactate, which points to a secondary generation of ROS prior to the NADH dependent generation of mitochondrial ROS. Following the initial decrease in

NAD^+ and NADH after 30 and 60 minutes, I found the levels of both oxidized and reduced NAD elevated after 2 and 6h respectively (Figure 4A).

A possible fate of lactate is the oxidation to pyruvate via the LDH reaction, which will then enter the mitochondrial TCA cycle that generates a series of metabolic intermediates with the concomitant production of NADH molecules that fuel the ETC. I found two of these intermediates – acetyl-CoA and Citrate – to be elevated in activated $CD4^+$ T cells after exposure to 10mM sodium lactate (Figure 4C-D). The amount of acetyl-CoA was elevated 2-fold after 10, 30 and 60 minutes, whereas citrate steadily increased to up to 8-fold after 2 hours of lactate exposure.

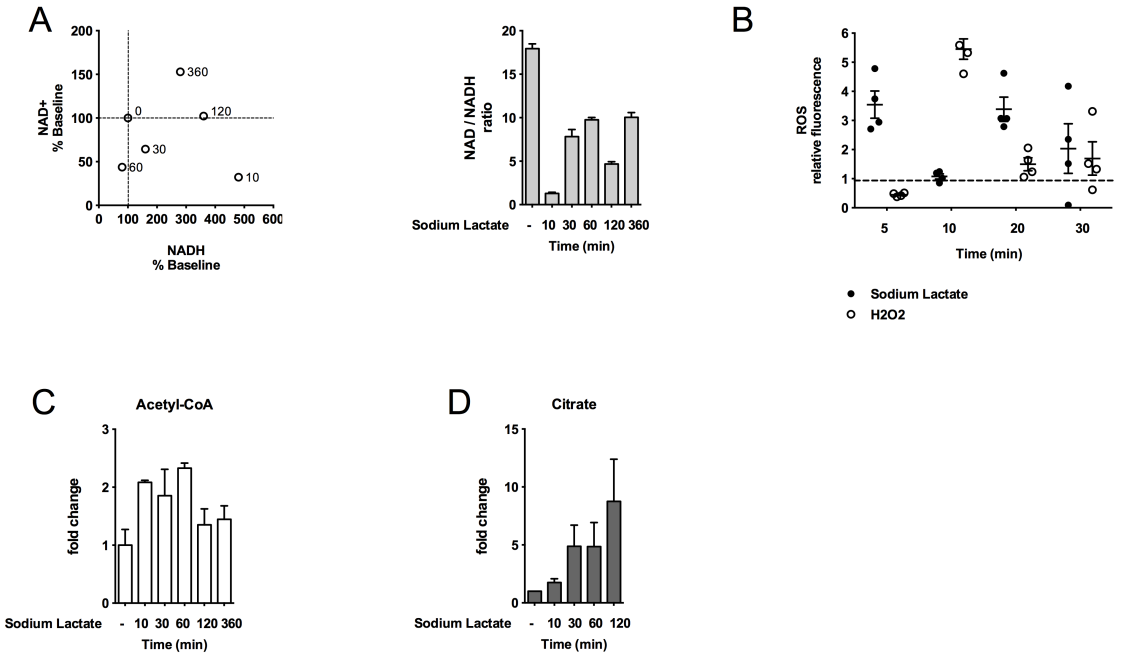


Figure 4 Lactate changes abundances of intracellular metabolites

Metabolite measurements of $CD4^+$ T cells exposed to 10mM Sodium Lactate for different time points in (**A**) NAD^+ / NADH depicted as % change compared to untreated (left) or ratio (right) and (**B**) Reactive oxygen species (ROS) compared to induction with 1mM H_2O_2. (**C-D**) Relative levels of acetyl-CoA (**C**) and Citrate (**D**) compared to untreated after Sodium lactate exposure. (**A-D**) Values denote mean ± SD. Combined data of four biological replicas; n=4. *P<0.05; **P<0.01; ***P<0.001.

5.2.2 Lactate decreases Glycolysis, Oxidative Phosphorylation and ATP levels

The redox couple NAD^+ / NADH as well as the metabolites acetyl-CoA and citrate are major regulators of cellular metabolism (Icard et al., 2012; Ying, 2008) and lactate itself has been shown to inhibit the rate limiting glycolytic enzyme Phosphofructokinase (Pfk) (Leite et al., 2011). To test the effect of extracellular lactate on metabolic activity, I measured glucose uptake and glycolytic flux using the fluorescent probes 2-NBDG and 6-NBDG in activated $CD4^+$ and $CD8^+$ T cells in the presence or absence of sodium lactate or lactic acid (Sukumar et al., 2013). Upon entering the cell 2-NBDG gets phosphorylated by hexokinase and rapidly degraded to non-fluorescent products. In contrast, 6-NBDG cannot be phosphorylated by hexokinase and accumulates in the cytoplasm in its fluorescent form. I could find that sodium lactate but not lactic acid selectively blocks glucose uptake and flux through glycolysis in $CD4^+$ but not $CD8^+$ T cells and vice versa, supporting the hypothesis of selective transporter dependent entry of lactate into these subsets (Figures 2A and 5A).

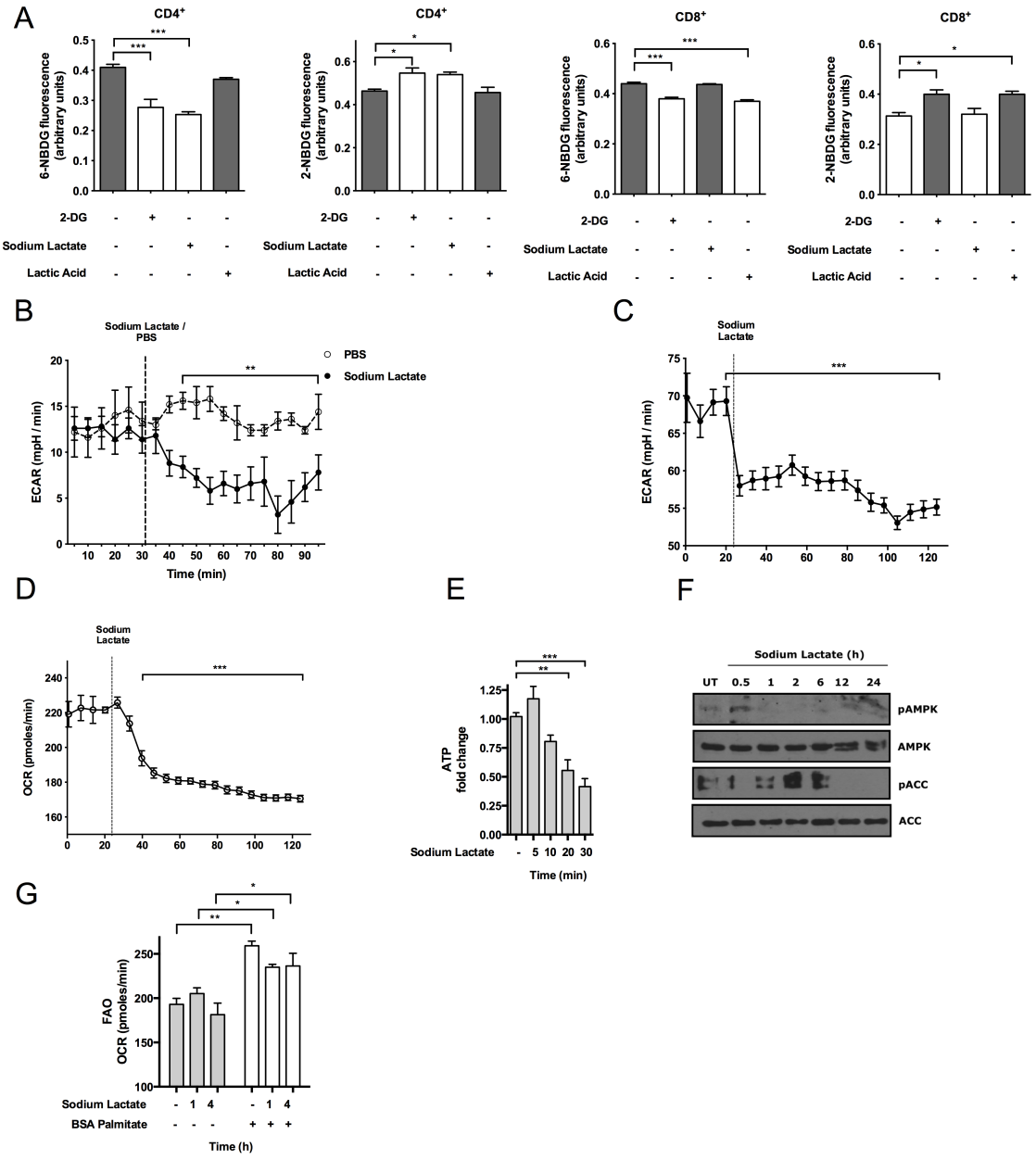


Figure 5 Metabolic changes induced by extracellular lactate

(**A**) Measurements of glucose uptake and flux in activated CD4$^+$ and CD8$^+$ T cells pre-treated with 2-DG, sodium lactate or lactic acid and then incubated with the fluorescent probes 6-NBDG or 2-NBDG. (**B, C**) ECAR trace of glycolytic activity in activated CD4$^+$ T cells treated with CXCL10 (1000ng/ml) and sodium lactate (10mM) in basal conditions with residual Glucose (**B**) or in the presence of 10mM Glucose (**C**). Vertical lines represent the addition times of CXCL10, sodium lactate or PBS. (**D**) Oxygen consumption rate (OCR) measurements in the of activated CD4$^+$ T cells in the presence of 10mM Glucose and addition of 10mM Sodium Lactate (vertical line). (**E**) Relative levels of ATP compared to untreated after Sodium lactate exposure. (**F**) Time course western blots with antibodies against pAMPK, total AMPK, pACC and total ACC in activated CD4$^+$ T cells treated with 10mM sodium lactate or left untreated. (**G**) Fatty acid oxidation (FAO) in CD4$^+$ T cells in the presence of 2.5mM Glucose, 167µM BSA-palmitate or BSA alone. (**A-G**) Values denote mean ± SD. (**A, B, F**) Data is representative of three independent experiments; n=3. (**C-E, G**) Combined data of four biological replicas; n=4. *P<0.05; **P<0.01; ***P<0.001.

To support these data, I measured glycolytic flux with the seahorse extracellular flux analyzer in basal conditions with only minimal residual glucose present as well as in the presence of 10mM glucose. In both conditions I could find that injection of sodium lactate decreased the extracellular acidification rate (ECAR) of $CD4^+$ T cells; in basal conditions from an average of 14 mpH/min in the untreated control to a level of less than 5mpH/min with lactate exposure (Figure 5B) and in the presence of glucose from 70mpH/min to 55mpH/min, confirming the observed decrease in glycolytic flux (Figure 5C).

As lactate has been shown to serve as a fuel for oxidative phosphorylation in a number of cells, including neurons and muscle cells (Cornell et al., 1973; Kompanje et al., 2007; Magistretti et al., 1994), I measured the OCR of $CD4^+$ T cells in the presence of 10mM sodium lactate, which is an indicator for mitochondrial activity. Surprisingly, also the OCR levels dropped from 220pmoles/min to 175pmoles/min within the first 20 minutes after injection, constituting a reduction of about 20% (Figure 5D).

Glycolysis and oxidative phosphorylation are two of the major ATP producing pathways in the cell. In line with the lactate-mediated inhibition of glycolysis and oxphos, also the ATP levels decreased 25% in the first 10 minutes and further fell down to 50% of the original value in the first half hour after lactate exposure (Figure 5E). A drop in ATP levels is a major activation signal for 5'-AMP activated protein kinase (AMPK), a master regulator for cellular homeostasis (Hardie et al., 2012). 30 minutes after sodium lactate exposure, I found AMPKα phosphorylated on Threonin 172 residue, which is a signal for its activation (Figure 5F). A downstream target of AMPK is the acetyl-CoA-Carboxylase (ACC), the rate-limiting enzyme for fatty acid synthesis (FAS) (Donaldson, 1979). Supporting the ATP and AMPK data, I also could detect phosphorylated ACC at Serin 79, up to 6h after lactate exposure, with no phosphorylation detectable at 12h and 24h (Figure 5F).

ACC phosphorylation inhibits its enzymatic activity and consequently the synthesis of fatty acids (Park et al., 2002). ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the main molecular precursor for fatty acid synthesis. When FAS is activated, malonyl-CoA also acts as an inhibitor for mitochondrial β-oxidation as it inhibits carnitine palmitoyl transferase 1a (CPT1a) by direct interaction and thus the transport of fatty acids into the mitochondrial matrix (Foster, 2012). I thus next tested whether lactate could also interfere with lipid metabolism and shift the metabolic program to the oxidation of fatty acids (FAO). I measured OCR in the presence of 2.5mM glucose and 1μM BSA-palmitate that served as a substrate for

FAO. The treatment with BSA alone reflects the oxphos mediated oxygen consumption, which I found comparable with previous measurements (Figure 5D and 5G) (Gerriets et al., 2015). The addition of BSA-palmitate raised the oxygen consumption significantly in the untreated and in the 4h sodium lactate treatment cells, indicating that the basal rate of exogenous FAO is not affected by lactate (Figure 5G). In order to measure the oxidation of endogenous fatty acids, I used the CPT1a inhibitor Etomoxir that blocks the uptake of FA (Schoors et al., 2015) into the mitochondria. Interestingly, no detectable difference between lactate treatment and control cells could be seen (Figure 5G).

To sum up, I could find that the presence of 10mM extracellular sodium lactate reduced the level of glycolysis, oxidative phosphorylation and consequently ATP levels, yet left fatty acid metabolism unaltered. It is tempting to conclude that high concentration of lactate represents a signal for the induction of FAO in order to rescue the metabolic breakdown of glycolysis and oxphos. This hypothesis is in accord with the recent detection of LDH in the peroxisomes, which represents a central organelle in fatty acid metabolism (Schueren et al., 2014).

5.2.3 Changes in global acetylation and PARylation patterns induced by sodium lactate

The availability of reduced and oxidized NAD has wide-reaching effects on cellular metabolism, as it is an essential cofactor of many biochemical reactions and so a central regulator of metabolic activity (Ying, 2008). One of the main NAD^+ dependent enzyme classes are the sirtuins, a family of histone deacetylases (HDACs), that are involved most prominently in regulating mitochondrial metabolism, aging, and mediate the effect of calorie restriction (Canto and Auwerx, 2009). Sirtuins most commonly ADP-ribosylate acetyl-lysine residues of acetylated proteins leading to their reversible deacetylation (Yang et al., 2006). The ability to deacetylate proteins is believed to be of great importance in the mitochondria, which contain abnormally high levels of NAD, which correlates with a relatively low number of acetylated proteins and normal mitochondrial function (Kim, 2006). In the absence of mitochondrial Sirt3, mitochondrial proteins become hyperacetylated, which leads in turn to mitochondrial dysfunction, which is a common phenomenon of age and -related diseases (Anderson and Hirschey, 2013). To test for activity of sirtuins, I probed whole cell protein lysates with an antibody that detects acetylated lysine residues. In the first 6h of sodium lactate treatment, I could not find any major changes in whole cell lysine acetylation, yet a shift in the band pattern occurred at later timepoints (12h, 24h) (Figure 6A).

Another class of NAD^+ dependent enzymes are the Poly (ADP-Ribose) polymerases that catalyze the polymerization of ADP-Ribose from NAD^+ onto a protein, attaching linear branched polypeptides (Kim et al., 2005). This PARylation plays significant roles in many cellular and molecular mechanisms, including DNA repair, apoptosis, chromatin modifications or mitosis (Kim et al., 2005).

To detect global changes in protein PARylation I used an antibody that detects ADP-ribose polymers. Interestingly, I again could find only minor changes in the PARylation pattern in the first 6h of lactate exposure, yet a complete absence of PARylated proteins upon 12h and 24h of treatment (Figure 6B). This absence of PARylation reflects lactate-induced inhibition of one or more PARP family members after 12h and 24h of lactate exposure (Kim et al., 2005).

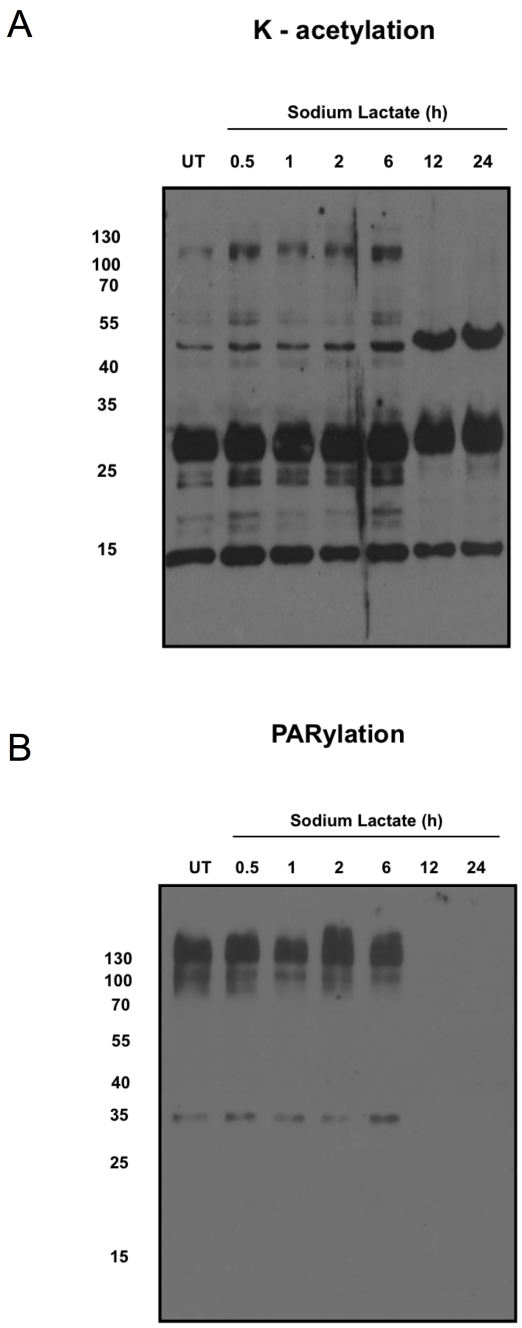


Figure 6 Sodium lactate induces changes in the cellular protein acetylation and parylation pattern.

(**A**) Western blot of whole $CD4^+$ T cell lysates exposed for different times to 10mM Sodium lactate and probed with antibodies against total Lysine-Acetylation (K-Ac) or PARylation (**B**). Data is representative of three independent experiments; n=3. Western blots in (**A**) and (**B**) are done on the same membrane.

5.3 CHAPTER III – THE BIOCHEMICAL BASIS FOR LACTATE-MEDIATED PHENOTYPE CHANGES

5.3.1 CXCL10 - Chemokine signaling induces Glycolysis in $CD4^+$ T cells

In order to find the metabolic basis and connect the biochemical alterations (Chapter II) to the observed defect in migration described in Chapter I, I started by investigating the effect of CXCL10 treatment on the induction of glycolysis in $CD4^+$ and $CD8^+$ T cells activated for 3 days with anti-CD3 and anti-CD28 antibodies and interleukin-2 (IL-2). I found that hexokinase 1 (HK1) and pyruvate kinase (PK) M2, two rate-limiting enzymes in the glycolytic pathway, were up-regulated at protein level in $CD4^+$ T cells at 2h and 4h after CXCR3 receptor engagement with CXCL10 respectively (Figure 7A). In contrast, $CD8^+$ T cells did not undergo major changes in the expression of glycolytic proteins upon exposure to CXCL10 (Figures 7B).

Additionally, mRNA levels of *Hk1*, *PkM2* as well as glucose transporters *Glut1-4* were also up-regulated 6h after treatment with CXCL10 in $CD4^+$ T cells, yet I could only find *Glut1* and *Glut3* up-regulated in $CD8^+$ T cells (Figures 7C and 7D) (Finlay, 2012). These findings suggest the existence of multiple regulatory mechanisms of the glycolytic pathway downstream of CXCR3 signaling, including transcriptional and post-translational control. Also, the discrepancy between $CD4^+$ and $CD8^+$ T cells to up-regulate glycolytic enzymes after CXCR3 engagement, points towards a differential regulation and metabolic requirement for chemokinesis in these subsets (Wahl et al., 2012).

Surprisingly, treatment of $CD4^+$ T cells with CXCL10 in the presence of sodium lactate resulted in the reduction of HK1 levels as compared to $CD4^+$ T cells treated with CXCL10 alone (Figure 7A). This effect could not be observed in $CD8^+$ T cells treated with CXCL10 and lactic acid, supporting the notion of differential metabolic requirements and control of migration (Figure 7B).

Glycolytic metabolism has recently been shown to be able to respond within milliseconds of engaging an extracellular signal (Barros et al., 2013) and these rapid changes are commonly regulated by enzymatic activities (Berg et al., 2007). I therefore tested whether CXCL10 also increases the level of glycolytic flux upon engagement with its receptor CXCR3. Exposing $CD4^+$ T cells to the chemokine raised the ECAR value from 13mpH/min to about 18mpH/min, indicating that the glycolytic flux is increased in these conditions (Figure 7E). I then asked whether the presence of sodium lactate was able to diminish this glycolytic increase also upon CXCR3 engagement by CXCL10, similarly to what had I observed previously

(Figures 5A-C) (Sidahmed et al., 2012). Adding sodium lactate to the CXCL10-stimulated cells decreased basal glycolysis to similar levels as detected with sodium lactate alone (Figure 5B), as reflected by a drastic fall of ECAR (Figure 7E). This suggests a dominant effect of lactate over CXCR3 signaling. The oxygen consumption rate was not affected by the addition of the CXCR3 ligand, yet it decreased about 25% within the first 20 minutes after sodium lactate treatment, confirming some of my initial findings (Figures 7F and 5D).

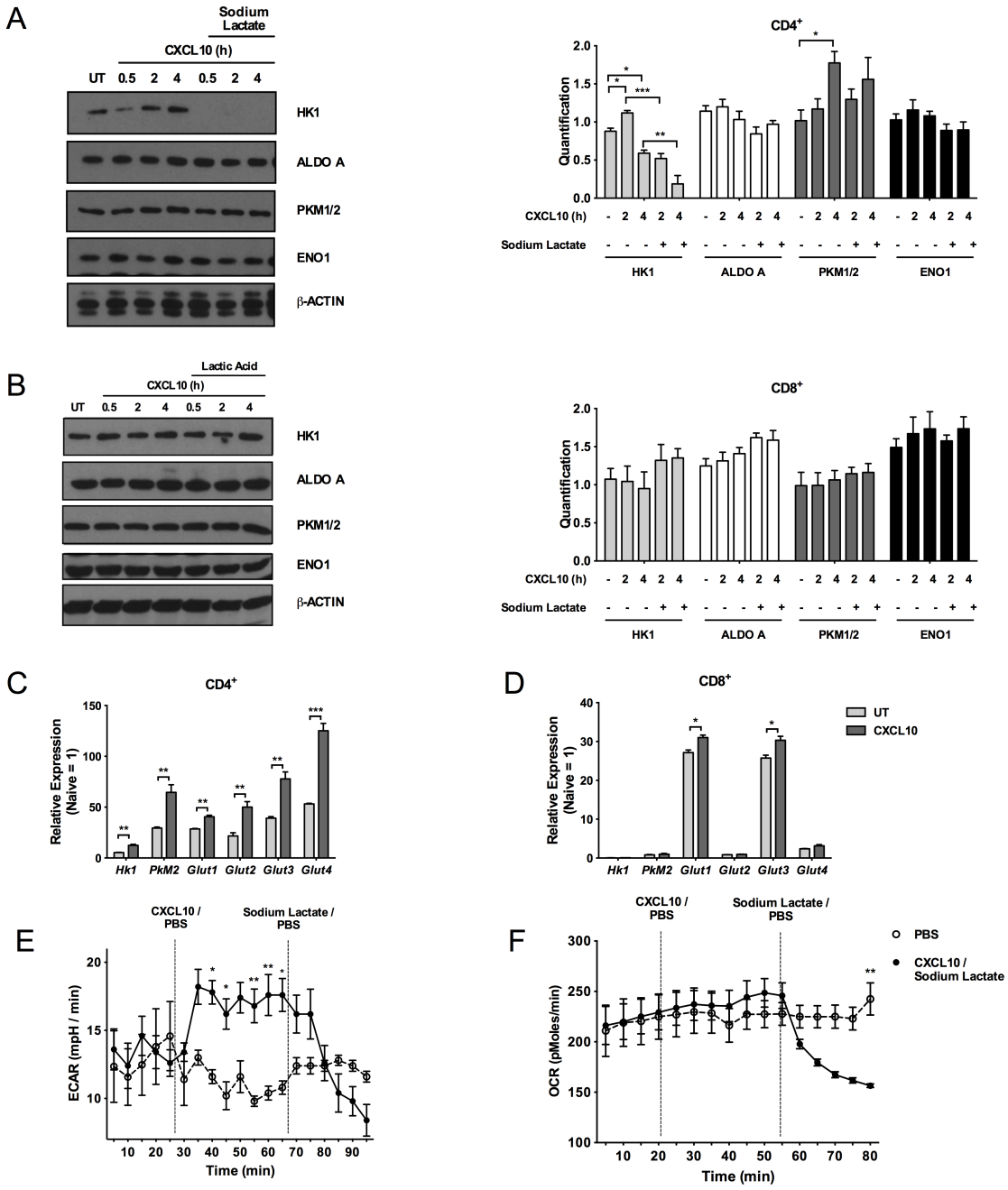


Figure 7 CXCL10 induces glycolysis

(**A, B**) Western blots with antibodies against HK1, PKM1/2, ALDO A, ENO1 and β-ACTIN in activated CD4$^+$ (**A**) or CD8$^+$ T cells (**B**) treated with CXCL10 (1000ng/ml) alone or in combination with sodium lactate (10mM) (**A**), lactic acid (**B**), or left untreated. (**C, D**) Relative mRNA expression levels of *Hk1*, *PkM2* and glucose transporters (*Glut1*, *Glut2*, *Glut3*, *Glut4*) in activated CD4$^+$ (**C**) or CD8$^+$ (**D**) T cells 6-hours post-treatment with CXCL10 (1000ng/ml) as assessed by qRT-PCR. mRNA levels in naïve, resting CD4$^+$ T cells were set to 1. (**E**) ECAR trace of glycolytic activity expressed as mpH/min in activated CD4$^+$ T cells treated with CXCL10 (1000ng/ml), sodium lactate (10mM) or PBS. (**F**) Corresponding OCR trace of CD4$^+$ T cells treated with CXCL10 (1000ng/ml), sodium lactate (10mM) or PBS. Vertical lines represent addition times of CXCL10, sodium lactate or PBS. (**A, B**) Densitometric quantification of western blots denotes mean ± s.e.m., n=3 (biological replicates, each run in duplicate). (**C-F**) Data is representative of three independent experiments. *P<0.05; **P<0.01; ***P<0.001.

5.3.2 Basal and chemokine-induced glycolysis is required for CD4$^+$ T cell migration both *in vitro* and *in vivo*

The finding that sodium lactate inhibits both glycolysis and oxidative phosphorylation in CD4$^+$ T cells (Figure 5A-E) but has no effect on FAO (Figure 5G) and CXCR3 signaling induces glycolysis specifically (Figure 7A-C and 7F), suggests that glycolysis is required for CD4$^+$ T cell migration.

To test this hypothesis, I treated activated CD4$^+$ T cells with inhibitors or activators of glycolysis and assessed their chemokinetic responses to CXCL10. Direct or indirect inhibition of glycolysis with the glucose analogue 2-DG or mTOR inhibitor rapamycin caused a decrease in chemokinesis *in vitro* (Figure 8A) and in a well-established *in vivo* model of T cell recruitment to the peritoneum (Figures 8B and 8G) (Jarmin et al., 2008).

Conversely, activation of glycolysis using the electron transfer chain Complex I inhibitor and AMPK activator metformin (Figure 8E and 8F) (Buzzai et al., 2007; Delmastro-Greenwood and Piganelli, 2013; Doherty et al., 2014; Yi et al., 2013), increased chemokinesis to CXCL10 both *in vitro* and *in vivo* (Figures 8A and 8B). Accounting for the specificity of the glycolytic measurements, etomoxir, an inhibitor of CPT1a and hence FAO, had only minor effects on glycolysis (Figure 8E).

In addition, metabolic drugs interfering with glycolysis had similar effects on T cell motility in spontaneous migration assays that are independent of any pro-inflammatory chemokine stimulus, implying the role of this pathway in steady-state control of T cell migration (Figure 8C). Importantly, exposure to the various metabolic drugs at the concentrations used did not affect the T cell surface molecule phenotype and receptor expression (Figure 8H and 8I), supporting the hypothesis that cell intrinsic metabolic mechanisms regulate the migration of T cells.

The importance of aerobic glycolysis in the activation and function of T cells has been shown previously (Chang et al., 2013; Gerriets and Rathmell, 2012; Michalek et al., 2011), yet its potential impact on naïve T cell migration is still unexplored. I therefore assessed the influence of glycolysis on migration of naïve T cells, which mainly rely on oxidative metabolism for their homeostasis (Gerriets and Rathmell, 2012). Similar to what I had observed in activated T cells, inhibition of glycolysis via 2-DG and rapamycin resulted in a decrease in naïve T lymphocyte migration towards the homeostatic chemokines CCL19/21 (Figure 8D), suggesting a general

control of T cell migration via the glycolytic pathway. In contrast to activated T cells, however, exposure to metformin reduced naïve T cell migration (Figure 8F), indicating the existence of different metabolic checkpoints between naïve and activated T cells (Wang and Green, 2012).

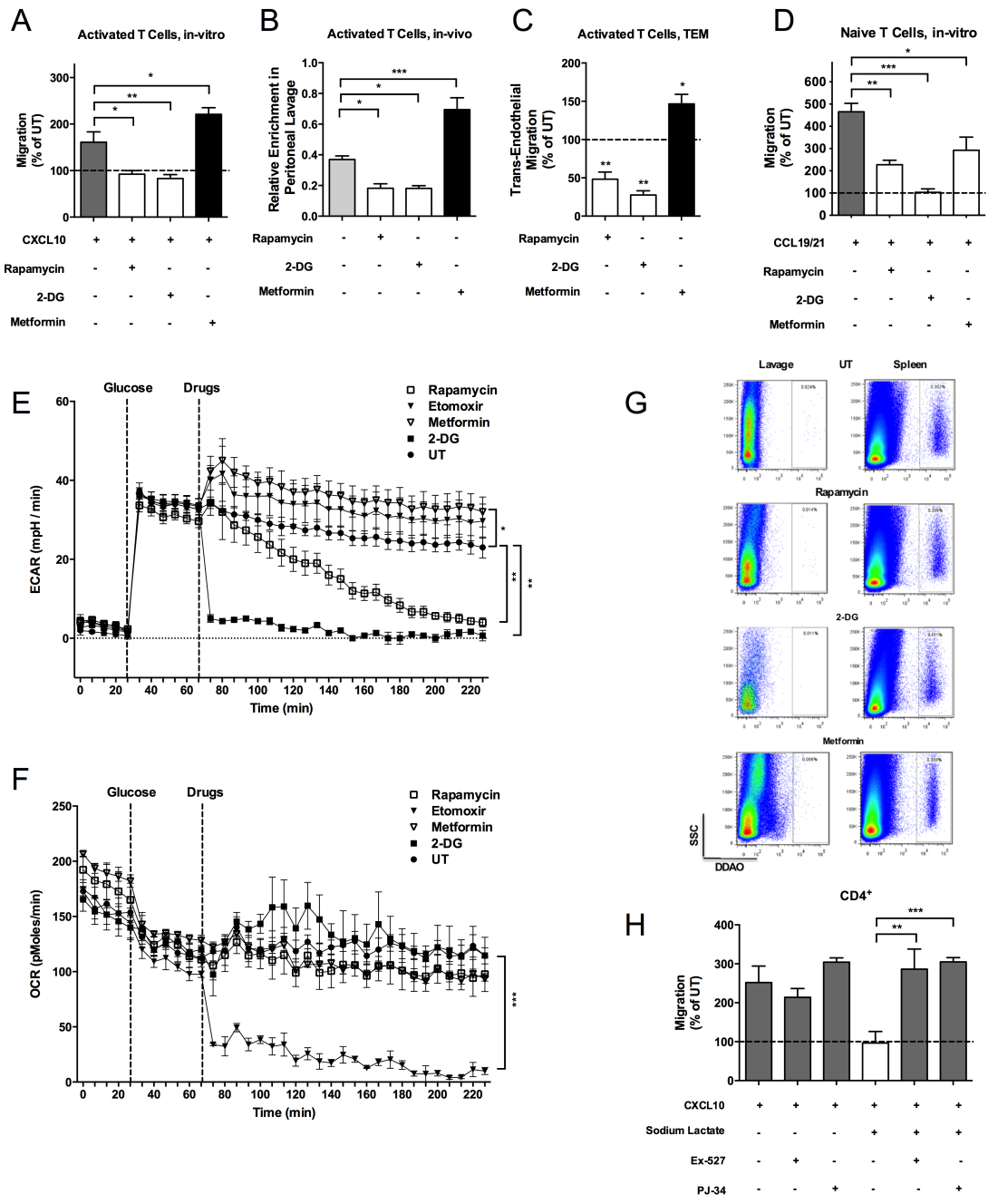


Figure 8 Basal and chemokine-induced aerobic glycolysis is required for CD4$^+$ T cell migration

(**A**) *In vitro* chemokinesis (4 hour time-point) towards CXCL10 (300ng/ml) of activated CD4$^+$ T cells pre-treated with Rapamycin (200nM), 2-DG (1mM) or Metformin (2mM). (**B**) Relative enrichment of i.v. injected activated CD4$^+$ T cells pre-treated with Rapamycin (200nM), 2-DG (1mM) or Metformin (2mM) and subsequently labelled with DDAO cell fluorescent dye in the peritoneal lavage of syngeneic recipient C57BL/6 mice i.p. injected with CXCL10 (120ng/mouse). (**C**) Spontaneous trans-endothelial migration (6 hour time-point) of activated CD4$^+$ T cells in the presence of rapamycin (200nM), 2-DG (1mM) or metformin (2mM).

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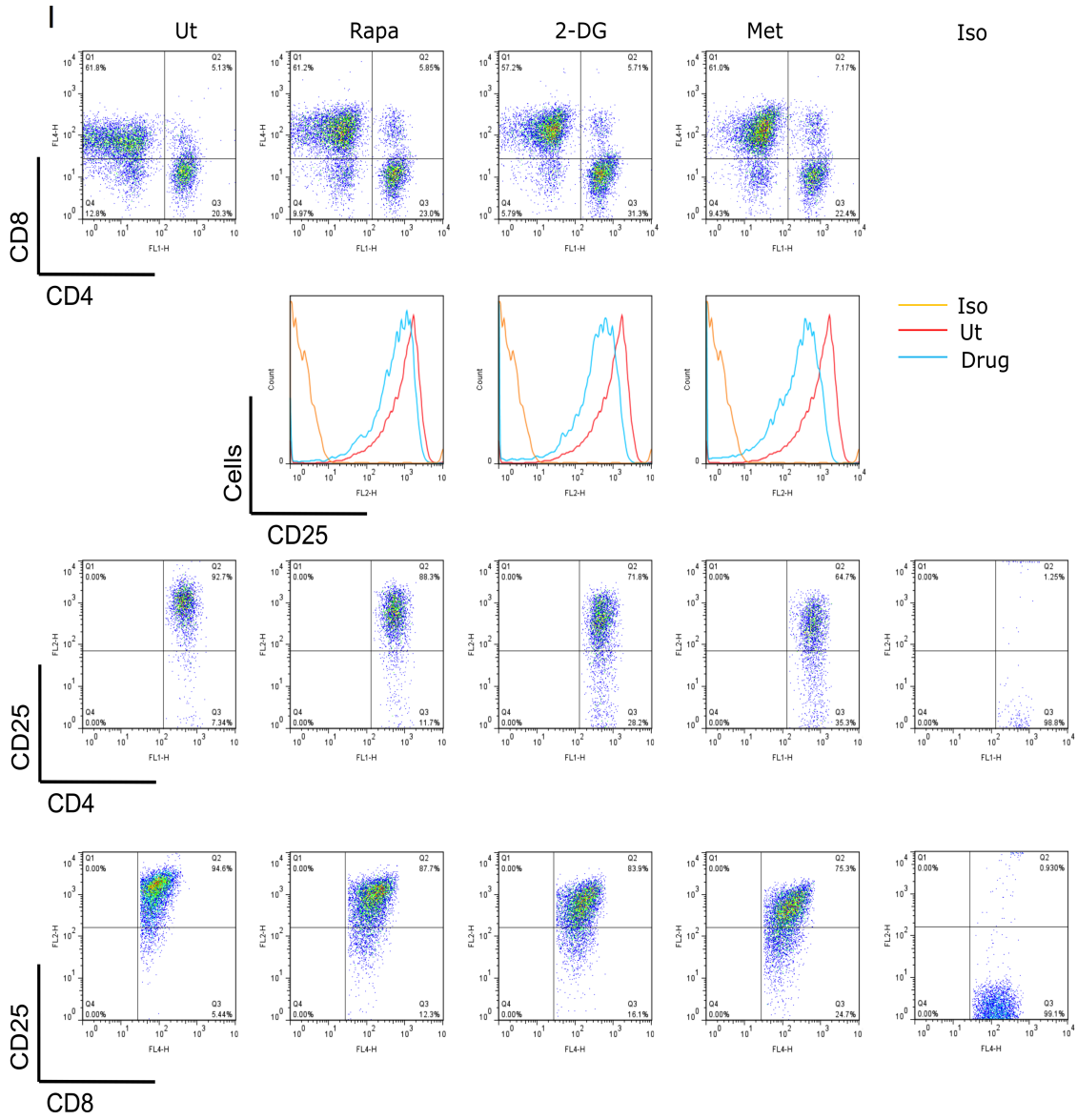

Figure 8 continued

(**D**) *In vitro* chemotaxis (4 h time point) towards the chemokines CCL19/21 (200 ng/ml of each chemokine) of naïve T cells pretreated with Rapamycin (200nM), 2-DG (1mM) or Metformin (2mM). (**E**) ECAR trace of glycolytic activity expressed as mpH/min and corresponding OCR trace (**F**) in activated $CD4^+$ T cells treated with 2-DG (1mM), Rapamycin (200nM), Metformin (2mM) or Etomoxir (40µM). (**G**) Representative FACS dot plots of DDAO-labelled donor $CD4^+$ T cells collected from the peritoneal lavage and spleen of recipient mice, which correspond to the relative enrichment in peritoneal lavage shown in Fig 8B. (**H**) *In vitro* chemokinesis (4 hour time-point) towards CXCL10 (300ng/ml) of activated $CD4^+$ T cells in the presence of sodium lactate pre-treated with Ex-527 (1µM) or PJ-34 (25µM).

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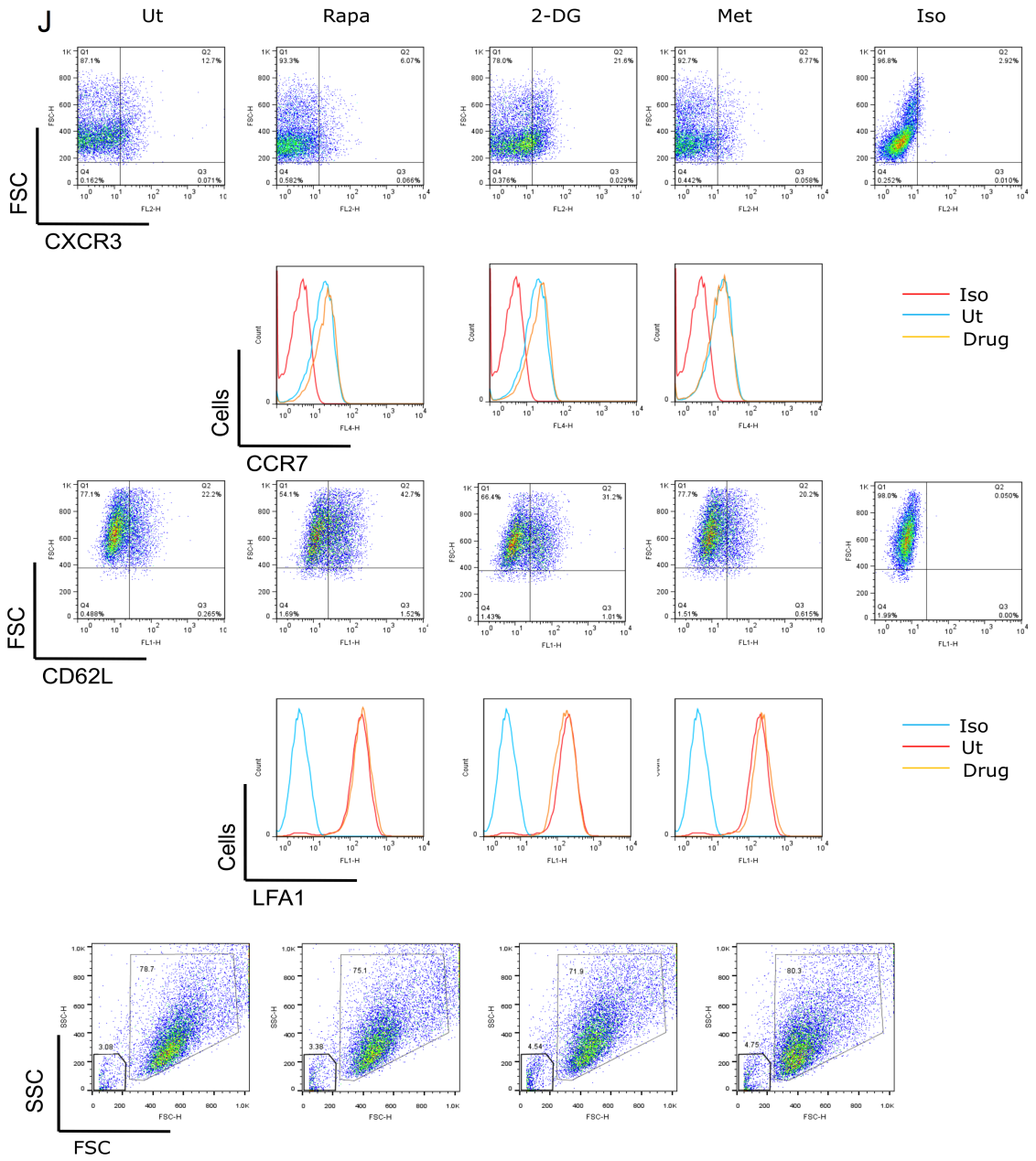

Figure 8 continued

(**I**) Representative FACS dot plots and histograms showing cell surface expression of CD4, CD8, and CD25 on activated T cells treated with Rapamycin (200 nM), 2-DG (1 mM) or Metformin (2 mM) as assessed by flow cytometry. (**J**) Representative FACS dot plots and histograms showing FSC/SSC profile and cell surface expression of CXCR3, CCR7, CD62L, and LFA-1 on activated T cells treated with Rapamycin (200nM), 2-DG (1mM) or Metformin (2mM) as assessed by flow cytometry. Data is representative of three (**A, C, D, H**) or two (**E, F**) independent experiments. (**B**) n=4 biological replicates. *P<0.05; **P<0.01; ***P<0.001.

To substantiate my hypothesis that glycolysis is necessary for the migration of activated $CD4^+$ T cells and to determine the relative contribution of NAD metabolism to the migratory phenotype (Hurd et al., 2012), I tested inhibitors of SIRT1 and PARP1, the major isoforms of sirtuins and poly (ADP-Ribose)

polymerases in the cell (Ba and Garg, 2011; Finkel et al., 2009). Ex-527 is a specific inhibitor of SIRT1 activity at the used concentration (Gertz et al., 2013) and PJ-34 is commonly used to inhibit PARP1 activity (Huang et al., 2008; Sajish and Schimmel, 2015). The sole inhibition of SIRT1 or PARP1 did not affect the chemokinesis in response to CXCL10, yet either of them could rescue the migration inhibition in the presence of sodium lactate (Figure 8H). This suggests that a crosstalk or a competition for NAD^+ between SIRT1 and PARP1 (Chung and Joe, 2014) could potentially exert the effects of the sodium lactate mediated migratory inhibition of $CD4^+$ T cells, dependent on glycolytic metabolism. A recent publication from Fouquerel et al. (2014) partly supports this hypothesis as they show that activation of PARP1 negatively regulates HK1 activity that causes a reduction in glycolysis and ATP levels, similarly to what I had observed (Figure 7A, 5A-C and 5E) (Fouquerel et al., 2014).

To conclude, I could find that glycolysis positively regulates the migration of $CD4^+$ T cells, which leads to the suggestion that the lactate mediated glycolytic decrease might as well be responsible for the observed migratory phenotype. Although the exact mechanism is not yet completely elucidated, the activity of the metabolic regulators SIRT1 and PARP1 seems to play a role. In this context it is interesting to notice that the activity of both SIRT1 and PARP1 have previously been linked to cell migration by interaction with cytoskeletal scaffold proteins (Cavone et al., 2011; Tang, 2010; Zhang et al., 2009).

5.3.3 Lactate exposure causes metabolic adaption in $CD4^+$ T cells

Next, I started to investigate how T cells adapt their metabolism in response to the exposure to extracellular lactate (Figure 5). First, I sought to confirm the sodium lactate-mediated decrease of HK1 protein (Figure 7A), which could provide an explanation for the detected decrease in glycolytic flux. I used confocal microscopy of lactate-treated $CD4^+$ T cells to assess the co-localization of HK1, mitochondria (MitoTracker) and nucleus (DAPI). Interestingly I could find that a 4 hour treatment with sodium lactate caused a relocation of HK1 from the cytosol to the mitochondria (Figure 9A) rather than a decrease in protein level (Figure 7A) (John et al., 2011). In order to investigate this discrepancy further I performed cell compartment isolation to enrich for cytosolic, mitochondrial and nuclear proteins of cells exposed to lactate with subsequent detection.

To analyse the purity of the enriched cellular compartments, I probed for the predominantly cytosolic, mitochondrial and nuclear proteins β-Actin, Pyruvate

Dehdrogenase (PDH) and Histone H3 respectively. Although I could detect some residual Histone H3 proteins also in cytosolic and mitochondrial fraction, the other isolated cell compartments were pure.

Confirming the microscopy data, I found a slight increase in mitochondrial enriched HK1 after 4 hour lactate compared to untreated cells (UT, Figure 9B left, John et al., 2011). A concurrent decrease in cytosolic HK1 levels in the lactate treated cells supports the hypothesis that lactate causes a translocation rather than degradation of HK1 to the mitochondria in line with previous observations (Neupert and Herrmann, 2007; Schindler and Foley, 2013). Interestingly, this translocation could only be detected for Hk1 and not another glycolytic enzyme PkM1/2 or the metabolic enzyme PARP1. Increased mitochondrial localization of HK1 is commonly associated with higher glycolytic flux that has been suggested to be due to increased availability of mitochondrial derived ATP (Wilson, 2003). Consistent with this hypothesis and confirming my previous data, I found glycolytic flux 4h after lactate treatment to be at the same level as in the untreated cells after an initial reduction in the first hour (Figure 9C).

Strikingly, 12 hours after lactate treatment the glycolytic flux was again reduced of about a third of the initial value, which aligned with a similar decrease in basal and maximum mitochondrial oxidative phosphorylation (Figures 9C and 9D).

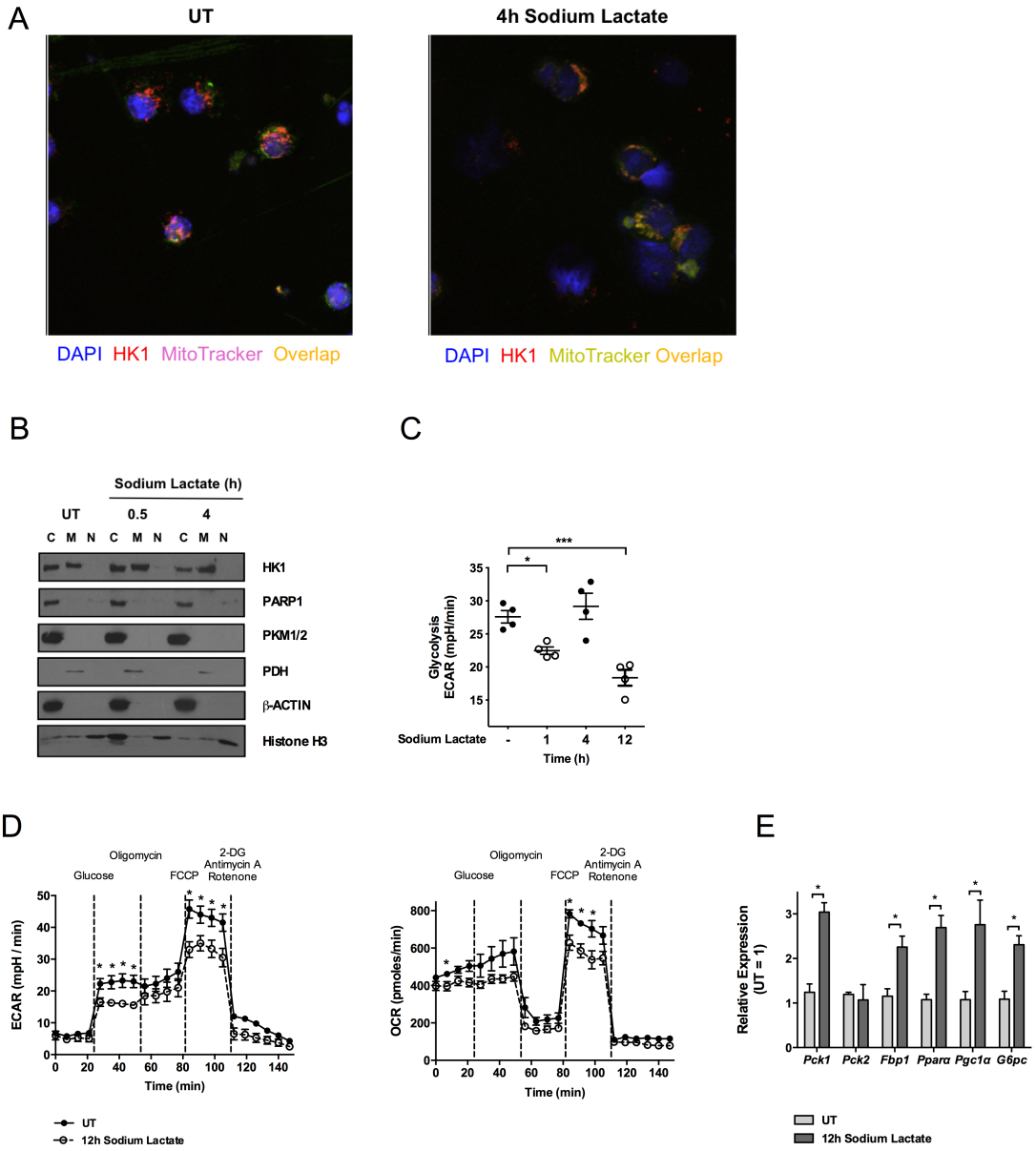

Figure 9 Metabolic adaption in CD4$^+$ T cells exposed to sodium lactate

(**A**) Confocal images of CD4$^+$ T cells untreated (left) or exposed to 4h sodium lactate (10mM), stained with DAPI, anti-HK1 antibody or MitoTracker Deep Red FM (300nM). (**B**) Western blots of cytosolic (C), mitochondrial (M) or nuclear (N) fractions of activated CD4$^+$ with sodium lactate (10mM), detected with antibodies against HK1, PARP1, PKM1/2, PDH, β-ACTIN and Histone H3. (**C**) Glycolytic ECAR measurement in mpH/min of CD4$^+$ T cells in the presence of 10mM Glucose and 10mM Sodium lactate. (**D**) Metabolic stress test analysis of CD4$^+$ T cells after 12h exposure to 10mM sodium lactate; ECAR trace (left) and OCR (right). (**E**) Relative mRNA expression of gluconeogenesis genes *Pck1, Pck2, Fbp1*, *Ppary*, *Pgc1α* and *G6Pc* in activated CD4$^+$ T cells exposed to 12h sodium lactate (10mM). mRNA levels in activated, resting CD4$^+$ T cells were set to 1. Data is representative of two (**A**) or three (**B**) independent experiments. (**C-E**) n=4 biological replicates. *P<0.05; **P<0.01; ***P<0.001.

Lactate can be utilized as a fuel source to generate glucose during the liver-muscle Cori cycle, so I next tested whether similar mechanisms might be at play here as

well. As reported in L6 muscle cells (Hashimoto et al., 2007), I could also find genes regulating gluconeogenesis up-regulated (Figure 9E). Amongst these were rate-limiting enzymes Pyruvate carboxykinase 1 (*Pck1*), Fructose-Bisphosphatase 1 (*Fbp1*), the glucose-forming enzyme Glucose-6-Phosphatase (*G6pc*) and the master transcription factors of gluconeogenesis *Ppara* and *Pgc1a* (Herzig et al., 2001; Patsouris et al., 2004; Yoon et al., 2001).

These data point towards a short-term metabolic adaption of $CD4^+$ T cells exposed to lactate to cope with the initial biochemical breakdown of glycolysis and mitochondrial oxphos and furthermore a long term adaptation towards gluconeogenesis in order to use extracellular lactate as an alternative fuel source (Cornell et al., 1973; Magistretti et al., 1994).

5.3.4 Sodium lactate induced IL-17 expression is dependent on NAD metabolism, FAS and Foxo1

Having established that a sodium lactate dependent decrease in glycolysis is the causative link to the observed migratory inhibition, possibly exerted via Sirt1 and Parp1, I next asked whether this glycolytic inhibition also causes the increase in IL-17 production (Figures 3A and 3B) (Shi et al., 2011; Yabu et al., 2011). I hence tested the effect of the glycolytic inhibitors 2-DG and Rapamycin as well as AMPK activator metformin on the production of IL-17. Surprisingly, both glycolysis inhibition and activation with the drugs caused a 75% increase in IL-17 protein secretion (Figure 10A middle), whereas sodium lactate only caused 40% more production (Figure 10A, left). These data suggest that the lactate-mediated inhibition of glycolysis is not the sole cause for the observed cytokine secretion.

As metformin is an inhibitor of the complex I within the ETC and only indirectly activates glycolysis via AMPK, which is activated due to a drop in ATP levels (Figures 5E and 5F) (Mihaylova and Shaw, 2011), I next tested the involvement of oxidative metabolism in the production of IL-17. Treating the cells with either the ATP synthase (complex V) inhibitor Oligomycin A (Shchepina et al., 2002), the mitochondrial uncoupler FCCP (Benz and McLaughlin, 1983), complex I or III inhibitors Rotenone (Li et al., 2003) and Antimycin A (Ma et al., 2011) respectively, did not cause any significant increase in the release of IL-17 (Figure 10A, right). These results indicate that the observed drop in oxphos after lactate exposure (Figure 5D) is not involved in the increased generation of the pro-inflammatory cytokine IL-17 and that additional parallel mechanisms might play a role.

To further identify the molecular pathways involved in the observed functional phenotype, I assessed whether the increase in IL-17 I could detect in the treatment with the glycolytic drugs (Figure 10A) is due to a transcriptional activation of the IL-17 gene. However, none of the used compounds showed a regulation at the mRNA level, which points towards a translational or post-translational mechanism in the regulation of 2-DG-, rapamycin- or metformin-mediated IL-17 secretion (Figure 10B, left) (Noubade et al., 2011) that is independent from the reported lactate induced effect (Figure 9B, left). Oxamate however, an additional glycolytic inhibitor that targets the LDH (Novoa et al., 1959), increased IL-17 mRNA on its own and rescued the sodium lactate dependent increase (Figure 10B, left), placing the biochemical reactions exerted by the LDH in the spotlight.

The involvement of LDH (Figure 10B, left) and the change in NAD^+ / NADH levels caused by sodium lactate (Figure 4A), led me to hypothesize that a lactate-induced change in the redox state leads to the production of IL-17. This hypothesis is supported by several reports that identify redox changes as regulators of cytokine production (Alam et al., 2010; Haschemi et al., 2012; Kesarwani et al., 2013). As previously, I used the SIRT1 inhibitor Ex-527 and the PARP1 inhibitor PJ-34 to test for their involvement. I noticed that not exclusive inhibition of Sirt1, but inhibition in the presence of sodium lactate could rescue the phenotype (figure 10B, middle). Similarly, PARP1 inhibition could also rescue IL-17 production by sodium lactate, yet on its own imitated the effect seen by sodium lactate alone (Figure 10B, middle). These data infer that the sodium lactate mediated transcription of IL-17 requires the activation of SIRT1 that might be dependent on the inhibition of PARP1 (Bai et al., 2011), as I had observed previously (Figures 6a and 6B).

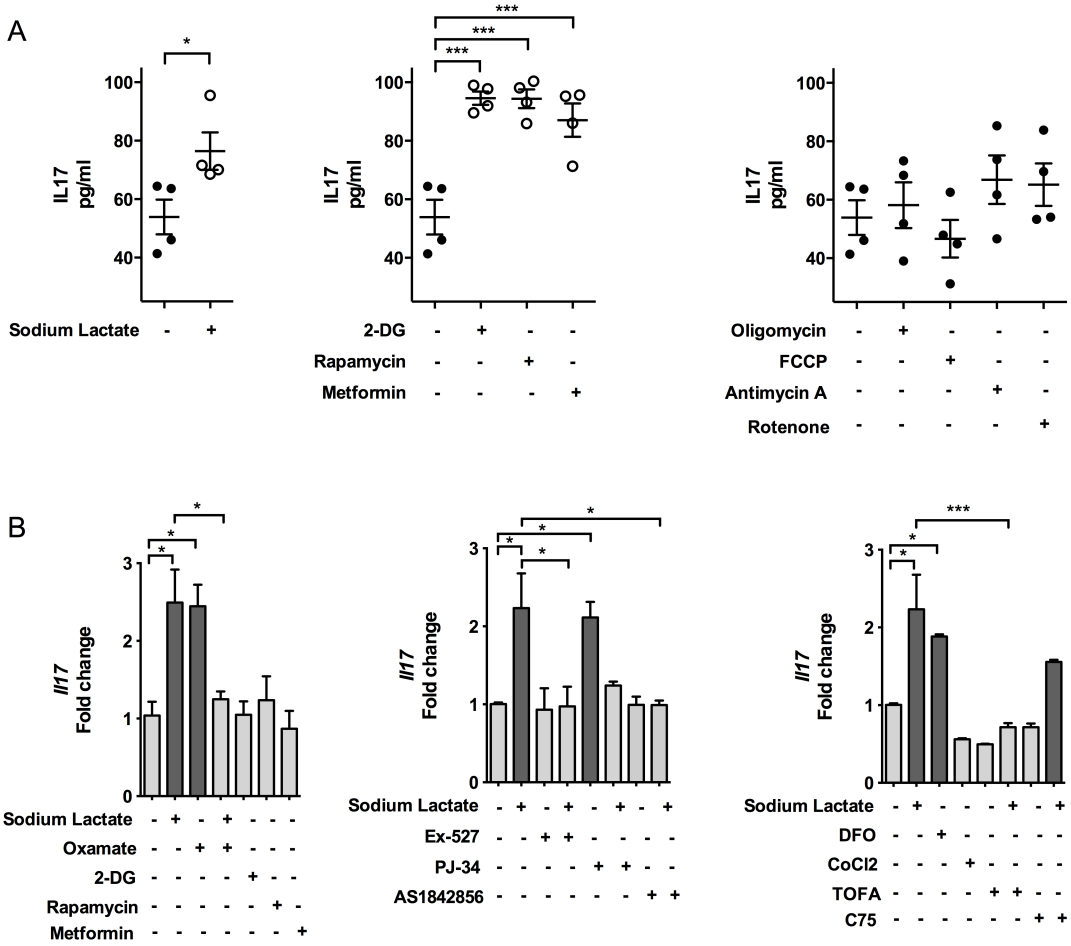

Figure 10 Lactate mediated IL-17 expression depends on NAD metabolism, FAS and Foxo1

(**A**) IL-17A ELISA from supernatants of activated $CD4^+$ T cells treated overnight with sodium lactate and metabolic drugs Rapamycin (200nM), 2-DG (1mM), Metformin (2mM). (**B**) Relative *Il17* mRNA expression of activated $CD4^+$ T cells treated with inhibitors or activators of glycolysis and oxphos (left), NAD metabolism and *Foxo1* (middle) or *Hif1α* (right) in the presence or absence of 10mM Sodium lactate. (**A, B**) n=4 biological replicates. *P<0.05; **P<0.01; ***P<0.001.

To find transcription factors that might be involved in the sodium lactate mediated IL-17 production, I tested the involvement of metabolic master regulators such as FOXO1, which has already been shown to influence the production if IL-17 and generation of Th17 cells (Ouyang et al., 2010). The FOXO1 inhibitor AS1842856 could normalize IL-17 mRNA levels in the presence of sodium lactate without showing effects on its own, proving the importance and involvement of FOXO1 in the lactate-stimulated IL-17 induction (Figure 10B) (Laine et al., 2015; Wu et al., 2013).

Extracellular lactate is a known inducer of hypoxia (De Saedeleer et al., 2012; Sonveaux et al., 2008), a cellular response regulated by HIF1α. Recently, HIF1α could

be shown to be indispensable for the generation of Th17 cells (Dang et al., 2011; Shi et al., 2011), so I investigated the possible role of this transcription factor in my settings. Two known inducers of hypoxia and HIF1α, Desferrioxamine (DFO) (Woo et al., 2006) and Cobalt Chloride (CoCl2) (Piret et al., 2002; Yuan et al., 2003) showed different effects on the induction of IL-17 (Figure 10B, right). Similarly, of the known Hif1α inhibitors TOFA and C75 (Berod et al., 2014), only TOFA could inhibit the induction of IL-17 mediated by sodium lactate (Figure 10B, right). These results point to the conclusion that although HIF1α has been shown to be important for the generation of TH17 cells (Shi et al., 2011) and the production of IL-17 in certain conditions (Dang et al., 2011), it possibly only plays a secondary role in the generation of IL-17 downstream of sodium lactate. Interestingly, TOFA activates Hif1α via inhibition of ACC, the rate-limiting enzyme of fatty acid synthesis (FAS) that I have earlier shown to be activated after 12h and 24h of lactate exposure (Figure 5F) and which has recently been reported to be essential for the induction of Th17 cells (Berod et al., 2014).

To sum up, I could exclude the direct involvement of either glycolysis or oxphos in the generation of sodium lactate dependent IL-17. I however identified FOXO1, ACC and SIRT1 activity as to be mediating the effects of sodium lactate on IL-17 expression. Implications and further direction will be discussed in a later chapter.

5.4 CHAPTER IV – THERAPEUTIC ASPECTS

Having established that accumulating extracellular lactate has widespread metabolic effects on T cells that lead to the inhibition of migration and secretion of the pro-inflammatory cytokine IL-17, I next interrogated the possible therapeutic implications of my findings.

5.4.1 Lactate signaling in Rheumatoid Arthritis

I have shown that in humans the synovial fluid of rheumatoid arthritis (RA; inflammatory form of arthritis) contains elevated levels of lactate compared to the non-inflammatory types (e.g. osteoarthritis [OA], Figure 1A). The rheumatoid synovial environment is paradigmatic of some of the lactate-induced changes seen in T cells, including entrapment, IL-17 secretion and loss of antigen responsiveness (Croia et al., 2013). I therefore investigated the expression and cellular localization of the lactate transporters SLC5A12 and SLC16A1 of 16 patients suffering from RA and undergoing total joint replacement therapy (Table 1, demographical data). I first stratified RA patients for the amount of $CD3^+$ infiltrating T cells using a semi-quantitative score (Figure 11A) as described in (Croia et al., 2013). Hereby, the CD3 score increases with the amount of T cell infiltrates.

I then performed gene expression analysis on total RA synovial tissue and found that *SLC5A12* mRNA expression significantly increased in correlation with the T cell score of the samples tested (Figure 11B). Albeit not significant, a trend towards increased *SLC16A1* expression could also be observed in $CD8^+$ T cells (Figure 11B, left).

In order to confirm my *in vitro* data that *SLC5A12* is expressed on $CD4^+$ but not $CD8^+$ T cells (Figure 2A), I performed double immunofluorescence microscopy for SLC5A12 and either CD4 or CD8. As shown in Figure 11C, SLC5A12 is abundantly and selectively expressed by $CD4^+$ but not $CD8^+$ T cells within the RA synovial tissue. Additionally, $CD4^+$ and $CD8^+$ T cells isolated from the peripheral blood of human healthy donors responded *in vitro* to sodium lactate and lactic acid similarly to their murine counterparts in terms of migration (Figure 11D) and expression of IL-17 protein (Figure 11E).

These findings establish lactate signaling as integral feature of RA and open up the possibility of targeting lactate for therapeutic intervention.

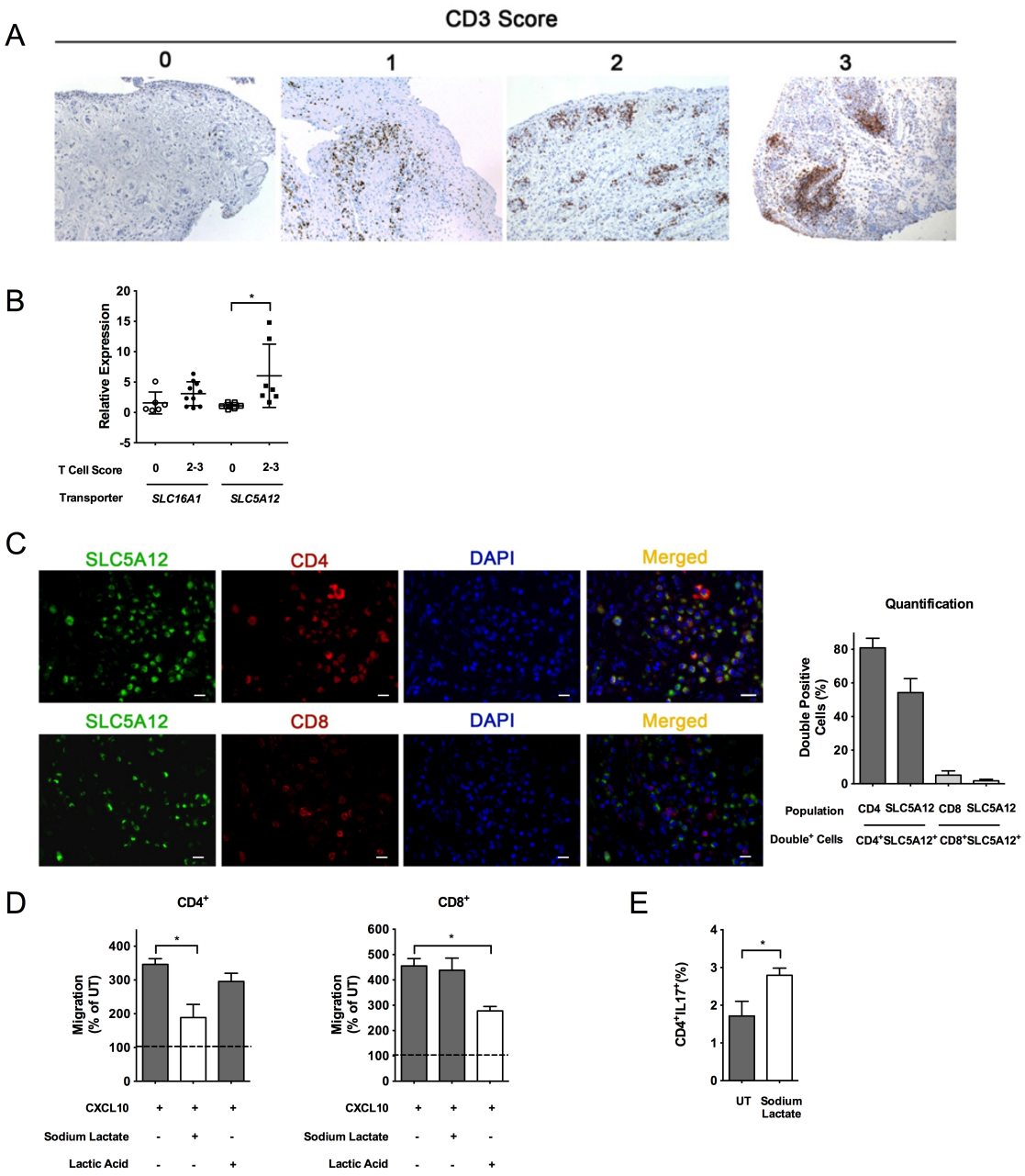

Figure 11 Lactate signaling in human RA

(**A**) Representative images of RA synovial tissues stained for CD3 displaying progressively higher degree of T cell infiltration as quantified using a semi-quantitative score from T0 (absence of infiltrating T cells) to T3 (large number of infiltrating T cells organizing in ectopic follicles). (**B**) Relative mRNA expression levels of *SLC16A1* and *SLC5A12* in the synovial fluid isolated from the joints of RA patients. Samples are grouped based on their T cell score as described in **A**. Values denote mean ± s.e.m., (T0) n=6 and (T2-3) n=9 (biological replicates, each measured in triplicate). *P<0.05. (**C**) Double immunofluorescence staining for SLC5A12 and CD4 or CD8 in the synovial tissue of RA patients. SLC5A12 (green) is highly expressed within the RA synovium in the presence of a high degree of $CD4^+$ (red) T cell infiltration. Merging (yellow) of the green and red channels demonstrates that SLC5A12 is selectively expressed by $CD4^+$ but not $CD8^+$ infiltrating T cells. Quantification of the % double positive cells is provided upon counting positive cells (single and double positive for each marker) in 6 images per condition. Columns represent % of double positive $CD4^+$ $SLC5A12^+$ population within the $CD4^+$ or $SLC5A12^+$ cells and % of double positive $CD8^+$ $SLC5A12^+$ population within the $CD8^+$ or $SLC5A12^+$ cells. Scale bars: 50 µm. (**D**) *In vitro* chemokinesis (4h time point) of activated human $CD4^+$ and $CD8^+$ T cells towards CXCL10

(300ng/ml) in the presence of lactic acid (10mM) or sodium lactate (10mM). (**E**) Intracellular staining of IL-17A in activated human $CD4^+$ T cells treated with sodium lactate (10mM) or left untreated. (**D, E**) Values denote mean ± s.e.m., n=4 (biological replicates, each measured in triplicate). *P<0.05; **P<0.01; ***P<0.001.

5.4.2 Inhibition of lactate transporters promotes the release of T cells from the inflamed tissue

I next sought to assess whether lactate promotes the retention of T cells in inflammatory sites *in vivo* and whether inhibitors of the lactate transporters favor the release of T cells from the inflamed site. I used a well-established mouse model of zymosan-induced peritonitis, in which T cells are recruited to the inflamed site 5 days after zymosan injection (Montero-Melendez et al., 2011). C57BL/6 mice were injected in the peritoneal cavity with zymosan (1mg/mouse) or left untreated. Five days later, Phloretin, an anti-SLC5A12 antibody or an isotype control antibody were injected into the peritoneal cavity. After 24h, mice were sacrificed and the peritoneal lavage was harvested. Lactate levels and $CD4^+$ and $CD8^+$ T cells were increased significantly in the peritoneum of recipient animals (Figures 12A and 12 B). Intraperitoneal injection of anti-SLC5A12 antibody caused a significant reduction of $CD4^+$ T cells in the peritoneum in comparison to an isotype-matched control antibody, while having no effect on $CD8^+$ T cells (Figures 12B and 12D-E). In contrast, phloretin promoted a significant decrease of $CD8^+$ T cells in the peritoneum but did not show any effect on $CD4^+$ T cells (Figures 12B and 12D-E).

To establish that the decrease in T cell localization to the peritoneal cavity was at least in part due to their increased release from this site, I performed adoptive transfer experiments whereby CFSE-labeled $CD4^+$ T cells were co-injected with anti-SLC5A12 antibody, phloretin or an isotype control antibody in the peritoneal cavity of mice which had been injected with zymosan (1mg/mouse) 5 days before, to create an environment rich of lactate (Figure 12A). 24 hours after the intra-peritoneal injection of CFSE-labeled $CD4^+$ T cells the mice were sacrificed and peritoneal lavage and spleen were harvested. I found that injection with anti-SLC5A12 antibody but not phloretin or the isotype control antibody in the peritoneal cavity caused a selective reduction of adoptively transferred T cells in the peritoneum and their accumulation in the spleen (Figures 12C and 12F).

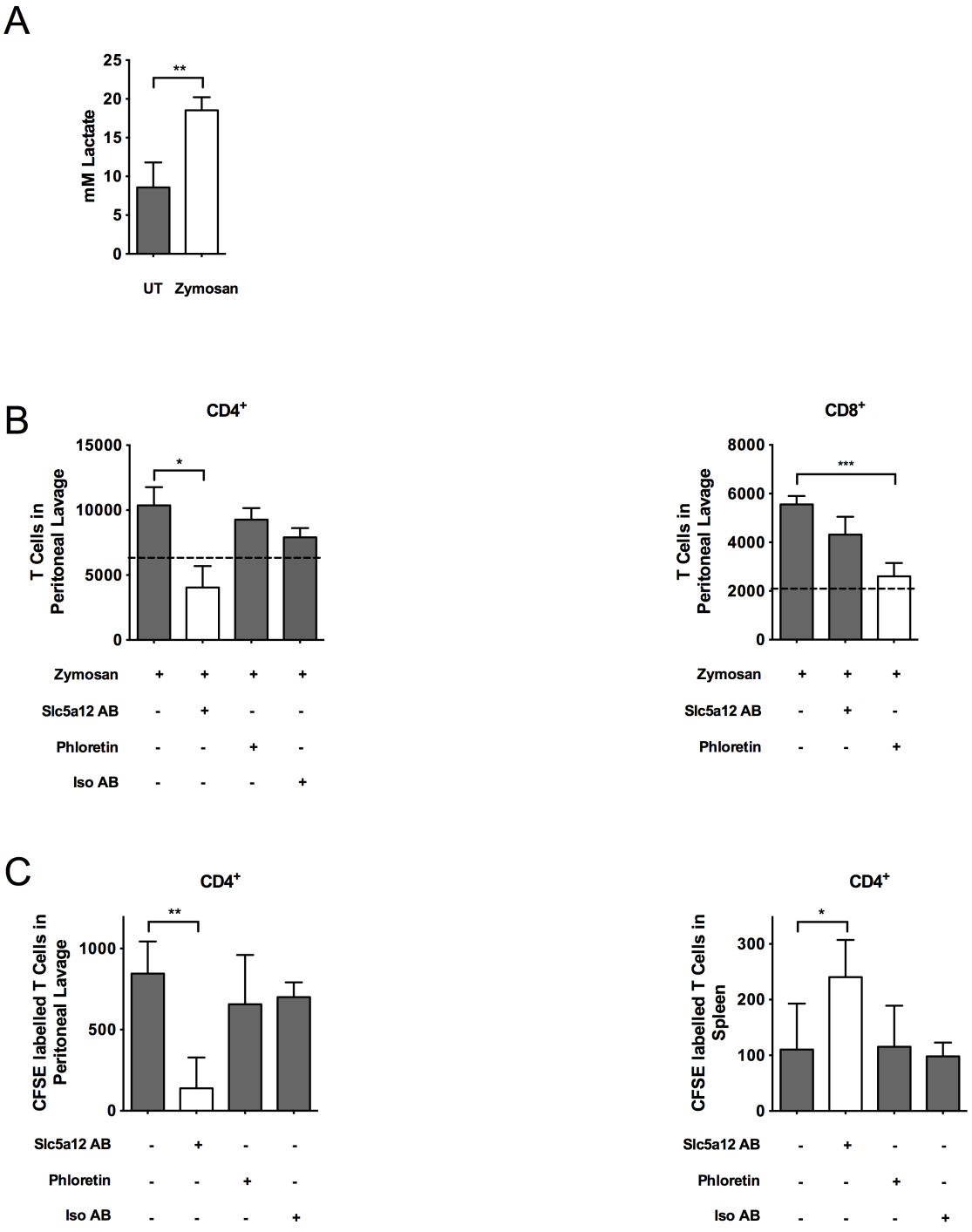

Figure 12 Inhibition of lactate transporters releases T cells from inflamed peritoneum

(**A**) Lactate levels in the peritoneum of zymosan-treated mice. (**B**) Number of $CD4^+$ and $CD8^+$ T cells, respectively, in the peritoneal lavage of C57BL/6 mice injected i.p. with zymosan (1mg/mouse) to induce peritonitis, and 5 days later i.p. treated with Phloretin (50µM), an anti-SLC5A12 antibody (5µg/ml) or an isotype control antibody. (**C**) Number of CFSE-labeled activated $CD4^+$ T cells in the peritoneal lavage (left panel) or spleen (right panel), respectively, of C57BL/6 mice injected i.p. with zymosan (1mg/mouse), then i.p. treated with Phloretin (50µM), an anti-SLC5A12 specific antibody (5µg/ml) or an isotype control antibody.

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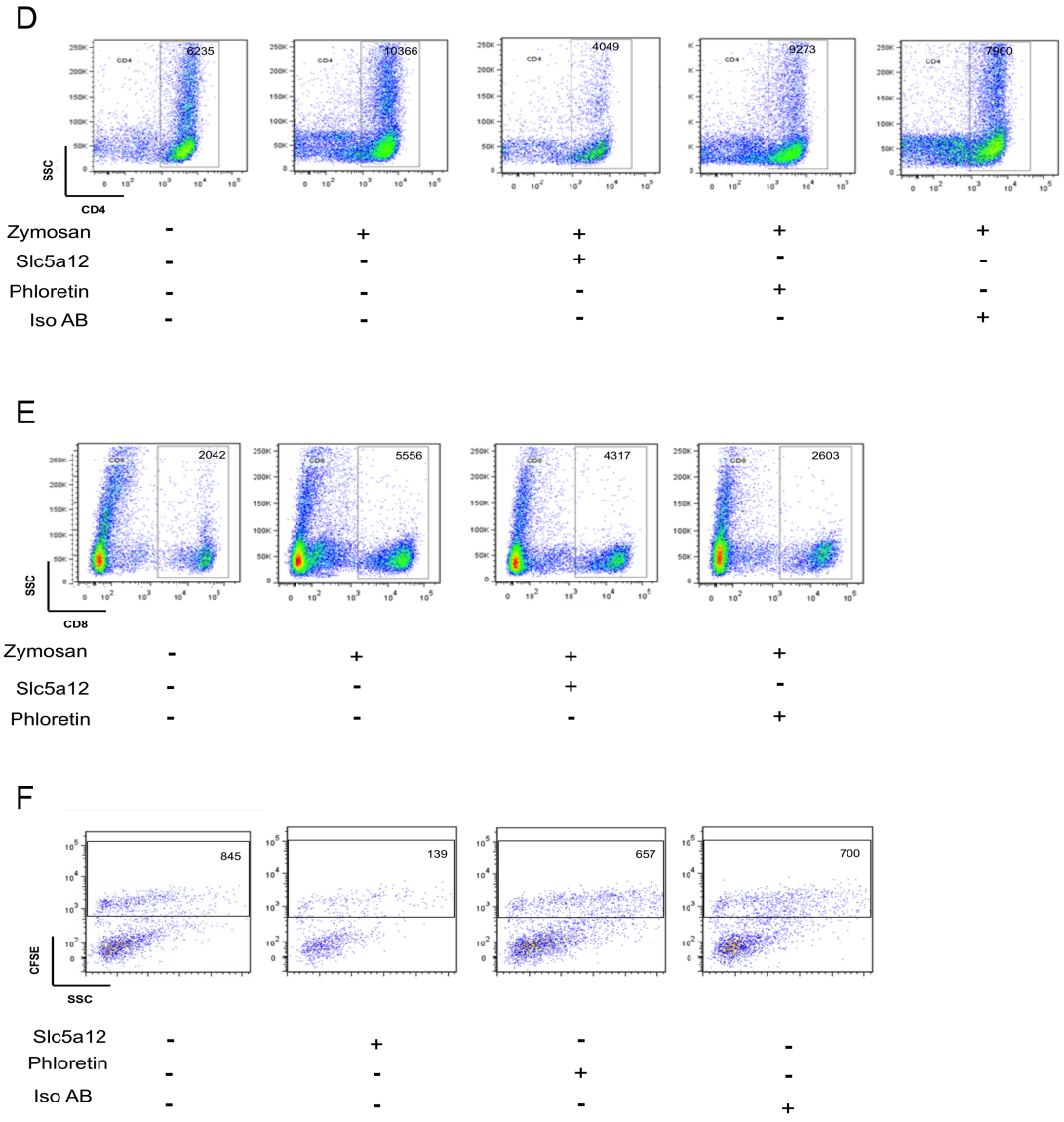

Figure 12 Continued

(**D, E**) Representative peritoneal lavage FACS dot plots of activated $CD4^+$ (**D**) and $CD8^+$ (**E**) T cells of C57BL/6 mice injected i.p. with zymosan to induce peritonitis, and 5 days later treated with SLC5A12 specific antibody (5µg/ml), phloretin (50µM) or isotype control antibody, which correspond to the $CD4^+$ and $CD8^+$ T cells in the peritoneal lavage shown in Figure 6B. (**F**) Peritoneal lavage FACS dot plots of adoptively transferred CFSE-labeled activated $CD4^+$ T cells, which are representative of the analyses shown in Figure 6C. Data is representative of three (**A, B, D, E**) and two (**C, F**) separate experiments. (**A-C**) Values denote mean ± s.e.m, n=3 (biological replicates). *P<0.05; **P<0.01; ***P<0.001.

6 DISCUSSION AND FUTURE DIRECTIONS

Chronic inflammatory diseases (CIDs) are a major public health concern in the western world. The non-resolving nature of inflammation in CIDs, associated with excessive and inappropriate activation of the immune system is critical for the disease process, yet some of the underlying cellular and molecular mechanisms have so far remained elusive. In this thesis, I have investigated the effect of lactate and the role of some major metabolic pathways in the regulation of T cell migration and functions in inflammation. Overall, I can draw four major conclusions of the above data that I will discuss in the context of recent literature.

6.1 LACTATE INHIBITS T CELL MIGRATION

Initially, I could show that the presence of 10mM extracellular sodium lactate or lactic acid inhibits the migration of $CD4^+$ and $CD8^+$ T cells, respectively, to a pro-inflammatory chemokine (Figure 1). This inhibition is mediated via two different short monocarboxylate transporters that are selectively expressed on each subset, which is a previously unknown difference.

The described transporters mediate the uptake of lactate, which then acts as a potent inhibitor of glycolysis affecting a number of mediators, including the rate limiting glycolytic enzyme phosphofructokinase (PFK) (Leite et al., 2011). Intriguingly, I found that the inhibition of glycolysis represents the underlying mechanism for the observed reduction in T cell migration. This finding is in line with a report that shows the critical involvement of the glycolytic activator Pfkfb3 in the reorganization of the cytoskeleton in endothelial cells (EC) (De Bock et al., 2013). Loss of PFKFB3 caused a decrease in proliferation and migration of ECs, necessary for the formation of filopodia and lamellipodia and subsequent vessel sprouting. In a further report the group could show that targeted glycolytic reduction via small molecule blockage of PFKFB3 resulted in reduced vessel sprouting in EC spheroids, zebrafish and murine retina, arguing for a widespread glycolytic control of cell migration (Schoors et al., 2015). Support for this hypothesis comes from the finding that reduced expression of the 11Beta-hydroxysteroid dehydrogenase type 1 (11βHSD1) mediates the induction of cell migration, invasion and angiogenesis via activation of glucose uptake and flux in hepatocellular carcinoma (HCC) (Liu et al., 2015). These findings stand in stark contrast to a previous report, showing that colorectal cancer cells rely on PGC1α dependent mitochondrial biogenesis, respiration and oxygen consumption for enhanced cell motility and cancer

invasiveness (LeBleu et al., 2014). Interestingly, using the small molecule Gamitrinib, Caino et.al (2013) could show that inhibition of the mitochondrial heat shock protein 90 (HSP90, TRAP1) also effectively reduced the migration of tumor cells (Caino et al., 2013). The inhibition or shRNA-mediated silencing of Tnf receptor associated protein 1 (TRAP1) resulted in a decrease in ATP levels and subsequent activation of AMPK, similar to the effects I had observed in $CD4^+$ T cells exposed to sodium lactate (Figure 5E and 5F). The phosphorylation of AMPK led to an inhibition of Focal adhesion kinase (FAK) and consequently disruption of Rho-Rac-Cdc42 mediated cytoskeletal rearrangement and cell migration. Contrarily to my findings in activated T cells treated with metformin (Figure 8A - 8C), constitutive active AMPK reduced tumor cell migration and metastasis in a mouse model of cancer metastasis (Caino et al., 2013). It is interesting to notice that in naïve T cells metformin had the opposite effect compared to activated T cells, pointing towards different metabolic requirements for cell migration or regulation thereof, possibly converging at AMPK, a master regulator for cellular homeostasis (Figure 8D; Wang and Green, 2012). More contradicting findings of AMPK promoting (Yan et al., 2015) or inhibiting cell migration (Nakano et al., 2010) possibly reflect the complexity of metabolic control of migration that is very likely cell type- and context-dependent and can be regulated by a wide variety of signals and effector molecules.

To establish a causative link between lactate and inhibition of CXCL10 induced chemotaxis, I investigated the involvement of the observed LDH coupled change in NAD^+ / NADH ratio (Figure 4A). The two major family representatives of NAD dependent enzymes Sirt1 and Parp1 are known to regulate cell migration, besides their role in controlling metabolic homeostasis (Cavone et al., 2011; Tang, 2010; Zhang et al., 2009). Interestingly, I could find that inhibition of either Sirt1 or Parp1 in the presence of sodium lactate could normalize the levels of chemotaxis towards CXCL10, yet had no effect on their own (Figure 8H). This finding suggests a lactate induced over activation and cross talk or competition for NAD^+ between Sirt1 and Parp1 in the regulation of migration (Fang et al., 2014). Strikingly, this hypothesis fits very well with a report that investigated the role of PARP1 in the developing *Drosophila* eye. Uchida et al. (2002) could demonstrate that transgenic flies overexpressing Parp1 showed a disturbance of tissue polarity due to a disruption of the F-actin cytoskeleton, a phenotype that strongly resembles the loss of function phenotype of the small GTPase RhoA (Strutt et al., 1997). They went on to identify a genetic interaction between RhoA and Parp1 in which co-overexpression of RhoA and Parp1 could partially rescue the observed effect of cytoskeletal disruption (Uchida et al., 2002). It will be interesting to investigate the exact mechanism of lactate mediated inhibition of $CD4^+$ T cell migration, the

relative contribution of metabolism and the molecular interactions of metabolic mediators with the cytoskeleton. It will be worth investigating the relative effects of lactate on mTORC2 signaling, which has been shown in neutrophils to control cell migration (Liu, 2010). The authors hereby describe a novel signaling network, in which mTORC2 regulates the production of cAMP through Adenyl cyclase AC9 and PKC. Production of cAMP in turn controls the activity RhoA GTPase, phosphorylation of myosin II at the rear end of the cell. Consequently, neutrophils inhibited in mTORC2 via knockdown of Rictor, show decreased ability of chemokine-induced cAMP production, cell polarization and rear end retraction. Interestingly, the authors could confirm that this effect was independent of actin polarization (Liu, 2010).

From the above data and reports it seems plausible that a cell uses various metabolic and molecular pathways to achieve migration that possibly depend on biochemical feasibility and the extracellular environment.

6.2 LACTATE CHANGES T CELL FUNCTION

As T cells are likely to be exposed to lactate as a consequence of activation and proliferation in the lymph node during an immune response (Buck et al., 2015), it is possible that the expression of either transporter determines the functional differentiation of T cell subsets (Sinclair et al., 2013). The physiological significance of this distinct transporter expression by activated $CD4^+$ and $CD8^+$ T cells might dictate their functional responses depending on the nature of the inflammatory exudate. As I could show in Figure 3 the presence of 10mM lactic acid or sodium lactate inhibits the cytolytic activity of $CD8^+$ T cells and induces the production of IL-17 in $CD4^+$ T cells, respectively. The observed inhibition of cytotoxicity in $CD8^+$ CTLs by lactic acid has previously been demonstrated by Fischer et al (2007), (Figure 3D). Interestingly, besides the inhibition of cytotoxicity that was due to a reduction in intracellular Perforin and Granzyme B abundance, also IL-2 and IFNγ production as well as proliferation was diminished. Importantly, these reductions were not due to a drop in pH conferred by the addition of lactic acid in the media, but the protons were required for lactate uptake in a MCT1-dependent manner (Fischer et al., 2007). As extracellular lactate is an inducer of hypoxia (De Saedeleer et al., 2012; Sonveaux et al., 2008), it is noteworthy that a recently published report describes the requirement of HIF1α in the expression of perforin in CTLs (Finlay et al., 2012). Although not directly investigated, it seems thus likely that the lactate-mediated reduction of CTL cytotoxicity is independent of hypoxia and Hif1α.

In the $CD4^+$ T cell subset, the exposure to 10mM sodium lactate induced the production of the cytokine IL-17 – associated to many inflammatory conditions - in all differentiation states (Figure 3A and 3B). These cells acquire a mixed phenotype whereby they continue to express their own signature cytokines but at the same time increase the transcription of RORγT and IL-17. In this context, it has recently been suggested that increased sodium chloride (NaCl) concentrations found locally under physiological conditions *in vivo* markedly boost the induction of murine and human Th17 cells in autoimmune conditions (Kleinewietfeld et al., 2013). The authors could show that elevated levels of NaCl stimulated the phosphorylation of p38 / Mapk, a known hypertonic stress response element, with concomitant activation of Nfat5 and its downstream target serum / glucocorticoid kinase 1 (Sgk1). Knockdown of either Sgk1 or Nfat5, or pharmacological inhibition of Sgk1, Nfat5 or p38 normalized the NaCl induced expression of IL-17 *in vitro* and *in vivo*. Interestingly, the Na^+ cation could be demonstrated to be responsible for the observed effect. These findings align with those reported by another group, which also found SGK1 as essential downstream effector of IL-23 signaling during the differentiation of Th17 cells (Wu et al., 2013). Using IL-17f^{Cre}Sgk1$^{fl/fl}$ knockout mice, in which SGK1 is specifically deleted in IL-17 producing $CD4^+$ T cells, the authors could show that the kinase does not affect primary Th17 differentiation, but rather affects their stability, provided by IL-23 signaling. Contrarily to both groups, which have used naïve $CD4^+$T cells, I have analyzed memory T cells already committed to a specific subset, similar to T cells in the inflammatory site and exposed them to another kind of salt – sodium lactate. Although not directly investigated, I found that the lactate ion rather than the sodium ion exerted the IL-17 induction via a LDH dependent mechanism (Figure 10B).

Lactate is known to inhibit glycolysis, at least in part via interaction with Pfk (Leite et al., 2011), yet glycolysis has been shown to be important for the differentiation of Th17 cells (Shi et al., 2011). Interestingly, both inhibition of glycolysis via 2-DG and Rapamycin, as well as activation via metformin could increase the secretion of IL-17 in activated, non-differentiated $CD4^+$ T cells (Figure 10A). Contrarily, the mRNA levels in these conditions did not change, which points towards a posttranscriptional mechanism that is independent to the sodium lactate mediated IL-17 induction. In line with this hypothesis, Noubade et al (2014) could show a posttranscriptional mechanism of IL-17 production that is mediated via the p38 / Mapk pathway, which is likely to represent the same stress pathway described earlier with NaCl (Kleinewietfeld et al., 2013; Wu et al., 2013). It seems thus plausible that the IL-17 production due to exposure of 2-DG, Rapamycin or

metformin is not mechanistically connected with the observed effect of sodium lactate, nor is it a direct consequence of glycolytic inhibition or activation.

Blocking glycolysis via the LDH inhibitor oxamate, however, could induce IL-17 mRNA synthesis to similar levels as those observed with sodium lactate, yet it rescued its sodium lactate dependent generation (Figure 10B). The LDH catalyzes the reduction of pyruvate to lactate with concomitant oxidation of NADH to NAD^+ when pyruvate is abundant or the generation of pyruvate when lactate is abundant. Downstream effector enzymes of NAD^+ include the histone deacetylase Sirt1 as well as the Poly-(ADP-Ribose) polymerase Parp1 (Chung and Joe, 2014). My results show that the inhibition of Sirt1 or Parp1 could reverse the effect of sodium lactate (Figure 10B), suggesting that decreased Parp1 activity is responsible for the transcription of IL-17. In line with this conclusion, Rutz et al (2015) found that inhibition of Parp1 with the small molecule Veliparib could increase the production of IL-17 in murine Th17 cells. Of note, the authors also showed an increase in *Rorc* and *Il17* mRNA, similar to the results I obtained with sodium lactate treatment (Figure 3). They further identified a Deubiquitinase DUBA – UBR5 dependent ubiquitinilation of RorγT that serves as a posttranscriptional break on IL-17 transcription (Rutz et al., 2015). The posttranslational regulation of IL-17 has recently received a lot of attention. Lim et al, (2015) show that the deacetylase Sirt1 promotes autoimmunity by deacetylating RorγT, which leads to the generation of Th17 cells and enhanced production of IL-17 cytokine. They found that Sirt1 physically binds to the C-terminal domain of RORγT and deacetylates three lysine residues in its DNA binding region, thereby enhancing its transcriptional activity. In a mouse model of experimental autoimmune encephalomyelitis (EAE), Sirt1 knockout mice or the inhibition of Sirt1 with the small molecule Ex-527 could protect the mice from the disease (Lim et al., 2015). In this context it is interesting to notice, that the inhibition of Sirt1 also normalized the IL-17 levels (Figure 10B). It seems thus likely that again Parp1 and Sirt1 act in an antagonistic crosstalk, whereby the inhibition of Parp1 is the crucial act that leads to IL-17 production (Chung and Joe, 2014; Rutz et al., 2015).

Besides the involvement of Parp1, I could also show that inhibition of Foxo1, a major transcription factor that controls cellular metabolism and has been implicated in Th17 cell differentiation (Laine et al., 2015; Ouyang et al., 2010), could normalize the levels of IL-17 mRNA upon lactate exposure. In contrast to my observations, Laine et al (2015) and Ouyang et al (2010) described FOXO1 as a negative regulator for Th17 differentiation. This discrepancy could be explained by the fact that the FOXO proteins function in response to environmental stimuli in order to ensure homeostasis (Eijkelenboom and Burgering, 2013). As the treatment

with sodium lactate represents a change in the cells' microenvironment, it seems plausible that also Foxo1 activity might adapt towards re-establishing homeostatic conditions. Similarly, I found that HIF1α, the major cellular response element to changes in environmental oxygen concentration, is not - or only partly - involved in the pathway that leads to the induction of IL-17 in response to lactate (Figure 10B). This finding is surprising, as both lactate is known to induce a state of pseudo-hypoxia (De Saedeleer et al., 2012; Sonveaux et al., 2008) and Hif1α has been shown to regulate Th17 differentiation in a variety of ways (Dang et al., 2011; Shi et al., 2011; Wang et al., 2014). Induction of hypoxia via DFO but not CoCl2 could raise *Il17* mRNA levels in a similar way to sodium lactate and inhibition of Hif1α with TOFA but not C75 could inhibit *Il17* expression in presence of sodium lactate (Figure 10B). Interestingly, TOFA is an inhibitor for ACC, the rate limiting enzyme of fatty acid synthesis (Donaldson, 1979), which has been shown to be important for the generation of Th17 cells (Berod et al., 2014).

In conclusion, the exact mechanism by which sodium lactate induces the transcription of *Il17* is not yet fully decoded, but I could show so far that it involves NAD metabolism via inhibition of PARP1 (Figure 6), FOXO1 activity and FAS. It will be interesting to investigate both transcriptional and potential post-translational effects that mediate the production of IL-17 in response to enriched lactate levels. The discrepancies between findings involving FOXO1 and HIF1α as regulator of Th17 cell differentiation are likely to reflect alternative adaption mechanisms to a changing microenvironment (Buck et al., 2015; Eijkelenboom and Burgering, 2013).

6.3 LACTATE CAUSES METABOLIC INHIBITION AND ADAPTION

Lactate has been shown to have a variety of effects on cellular metabolism, including the inhibition of glycolysis (Berg et al., 2002)the increase of mitochondrial oxphos in neurons (Magistretti et al., 1994; Tarczyluk et al., 2013), the induction of gluconeogenesis during the Cori cycle in the liver and the synthesis of glycogen in skeletal muscle cells (Cornell et al., 1973). Consistent with these reports, I observed a drastic decrease in glycolytic flux within the first 10 minutes after exposure to lactate, an indication for regulation at the level of enzyme activity (Zhao and Qu, 2009). This is in line with a previously described feedback inhibition of glycolysis by direct interaction of lactate with PFK, a rate-limiting enzyme of the glycolytic pathway (Leite et al., 2011). Interestingly, also the levels of oxidized NAD dropped within the first 10 minutes of lactate treatment, which is most likely due to

the reversal of the LDH reaction (Figure 4A, Equation 1). NAD^+ is used as an essential cofactor for the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reaction that catalyzes the conversion of D-glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. It remains an open question which of the proposed mechanisms of glycolytic inhibition is in play in $CD4^+$ T cells, if both are occurring simultaneously or even additional ways of inhibition exist (Williamson, 1965).

Interestingly though, the initial reduction of NAD^+ to NADH 10 minutes after lactate exposure was followed by a decrease in NADH levels at 30 and 60 minutes that was not due to re-oxidation to NAD^+, suggesting its possible utilization by the electron transport chain (Ying, 2008) (Berg, 2002). In line with this hypothesis, the levels of reactive oxygen species (ROS) in whole cell extracts 20 and 30 minutes after lactate exposure were increased (Figure 4B). Surprisingly, ROS levels also displayed an early increase after 5 minutes of lactate treatment. In this context, a recent report describes the identification of a putative flavin-dependent lactate oxidase in the intermembrane space of hepatic mitochondria (de Bari et al., 2010). The authors could demonstrate that lactate exposure to isolated mitochondria produced pyruvate in a 1:1 stoichiometry with the concomitant release of H_2O_2 that was independent from the respiratory chain. It is possible that a similar mechanism also led to the transient increase in ROS levels in $CD4^+$ T cells.

Mitochondrial ROS results from stepwise reduction of O_2 as a secondary product of the ETC, and acts as a signaling molecule in a variety of cellular responses (Chouchani et al., 2014; Murphy, 2009; Okoye et al., 2015; Sena et al., 2013). It is thus interesting to note that although lactate has been shown to be able to act as a fuel for oxidative metabolism (Magistretti et al., 1994; Tarczyluk et al., 2013) and to increase ROS production, $CD4^+$ T cell mitochondrial activity and ATP levels decreased after exposure to lactate (Figure 5D ad 5E).

The LDH dependent oxidation of lactate yields pyruvate, which can then enter the TCA cycle after being converted to acetyl-CoA. I indeed found levels of acetyl-CoA enriched 2-fold between 10 and 60 minutes after lactate treatment (Figure 4C). More strikingly, citrate levels were increased up to 8 fold (Figure 4D). An accumulation of citrate and succinate was recently reported during the differentiation of classically activated (M1-like) macrophages using an integrated high-throughput transcriptional-metabolic profiling approach (Jha et al., 2015). The authors identified a break of the TCA cycle after Isocitrate dehydrogenase (IDH), which converts isocitrate to α-ketoglutarate and was responsible for the citrate accumulation. In macrophages citrate can be used for the production of the antimicrobial agent itaconic acid or for the synthesis of fatty acids, a hallmark of M1

polarization (Infantino et al., 2011). Interestingly, after lactate treatment I also detected activation of the rate limiting enzyme for fatty acid synthesis acetyl-CoA Carboxylase (ACC) (Figure 5F), which has been shown to be indispensable for IL-17 production in $CD4^+$ T cells (Berod et al., 2014). Moreover, citrate is a known inhibitor of glycolysis and its accumulation might be responsible for the prolonged glycolysis inhibition observed (Newsholme et al., 1977; Williamson, 1965).

Surprisingly though, I found that the glycolytic activity normalized 4 hours after exposure to lactate (Figure 9C). In conjunction with the increased mitochondrial localization of HK1 at this time point (Figure 9A and 9B) this might reflect a metabolic adaption to rescue the glycolytic flux and ATP production (Calmettes et al., 2013). The localization of Hexokinases to the mitochondrial membrane is a widespread adaptive phenomenon in response to energy depletion. It is proposed to directly expose the enzymes to newly formed ATP from the mitochondria and thereby rescue the glycolytic flux in the cytoplasm. More in depth investigation is necessary to elucidate other metabolic pathways affected by the presence of lactate, adaption mechanisms and their consequences, and whether interference with these are able to reproduce or rescue some of the cellular effects observed (Berod et al., 2014; Uchida et al., 2002). Moreover, a broad scale metabolic analysis would also be able to reveal, whether lactate exposure could cause a break in the TCA cycle leading to citrate and succinate accumulation that is responsible for the reported cellular consequences.
A working model for the intracellular signaling pathways is presented in Figure 13.

6.4 LACTATE SIGNALING PERPETUATES CHRONIC INFLAMMATION

The phenotype of T cells exposed to lactate recapitulates key features of T-lymphocytes found in chronic inflammatory infiltrates, including their entrapment, the production of high levels of IL-17 production by $CD4^+$ T cells and loss of cytolytic activity in $CD8^+$ T cells (Figure 14A) (Gerriets and Rathmell, 2012; Haas et al., 2013). I could show that sodium lactate induces all $CD4^+$ Th subsets to increase their production of the pro-inflammatory cytokine IL-17 via a not yet completely elucidated mechanism, which is prevalent in the inflamed tissue and is known to drive forward the chronic inflammatory process in many CIDs (Gerriets and Rathmell, 2012; Haas et al., 2013). I hence tested the significance of lactate signaling in a disease model of chronic inflammation by investigating human samples of rheumatoid arthritis. In keeping with the *in vitro* data, I found that in chronically inflamed synovial tissue, a disease associated with high levels of IL-

17^+/$CD4^+$ T cells in the joints (Chabaud et al., 1999), infiltrating $CD4^+$ but not $CD8^+$ T cells almost invariably displayed high expression of the lactate transporter SLC5A12. This suggests that the expression of SLC5A12on the $CD4^+$ subset may be involved in the entrapment of $CD4^+$ T cells within the chronically inflamed "milieu" of the RA synovium (Figure 14A). I could further demonstrate that targeting the lactate transporters SLC16A1 and SLC5A12re-establish T cell migration to consequently release them from the inflammatory site and block the production of IL-17, with potential therapeutic implications in the management of CIDs (Figure 13B). In this context, it has been shown that the neutralization of IL-17 protein in a mouse model of Collagen induced arthritis (CIA) could reduce joint inflammation, cartilage destruction and bone erosion (Lubberts et al., 2004). In rheumatoid arthritis a combined treatment of dual cytokine inhibition (IL-17 and TNFα) shows greater potential than single neutralization itself (Buckland, 2014; Fischer et al., 2015). In light of this, interference of lactate signaling in RA by inhibition of the two identified transporters paves the way for additional therapeutic approaches. Hereby, targeting not only the production of IL-17, but also the entrapment of T cells in the inflamed site represents a potential treatment for two independent mechanisms of disease progression (Al-Saadany, 2015; Haas et al., 2013). It should be borne in mind, however, that the effect of IL-17 in other models of chronic inflammation is however still controversial, as IL-17A or IL-17F gene knockout in a model of autoimmune neuro-inflammation did not alter any of the pathological signs (Haak et al., 2009).

Additional evidence has to be generated to define the functional effects of Slc16a1 inhibition on the $CD8^+$ T cell subset in CIDs. Interestingly, the role of $CD8^+$ T cells in RA is not completely understood. On the one hand, activated $CD8^+$ T cells express high levels of the pro-inflammatory cytokines IFNγ and TNFα that drive RA associated pathologies and have been shown to regulate the structural integrity of the synovial membrane (Kang et al., 2002; Wagner et al., 1998). On the other hand, RA is often associated with persistent viral infections that inversely correlate with the function of virus-specific $CD8^+$ T cells in the synovial fluid and worsened disease outcome (Croia et al., 2013). In this context, the presence of enhanced lactate levels in the inflamed synovial fluid is likely to impact some of the $CD8^+$T cell-mediated pathological features of RA and represents another interesting target for therapeutic intervention.

In summary, my observations suggest that lactate signaling in the inflammatory "milieu" may promote some of the pathogenic features and persistence of the T cell infiltrates. Importantly, I show that pharmacologic targeting of lactate transporters

in T cells might provide a novel approach to resolve chronic T cell-mediated inflammation in CIDs.

6.5 FUTURE DIRECTIONS

Although in this thesis I could shed some light on novel aspects of lactate signaling in chronic inflammation, several key questions remain unanswered that should be addressed in the future.

I could demonstrate, that the effect of extracellular lactate on T cell migration and function is mediated via transmembrane transporters, selectively expressed on $CD4^+$ and $CD8^+$ T cell subsets (Figure 2). However, the actual uptake of lactate in T cells has not been assessed. The most common techniques to measure direct uptake of a metabolite by a cell includes treatment with a radioactive isotope and subsequent determination of intracellular radioactivity in a cell lysate, as could be achieved with L-(U-14C) lactate. Another, more detailed method includes the use of a stable isotope with one or more 13C atoms in conjunction with either mass spectrometry or nuclear magnetic resonance spectroscopy to delineate the specific intracellular fate of lactate. This technique would provide important mechanistic insights of lactate action.

Additionally it will be important to determine the overall intracellular signaling network of lactate, including the investigation and biochemical characterization of lactate transporters and other lactate utilizing enzymes in the different subcellular compartments (Schueren et al., 2014; de Bari et al., 2010) of $CD4^+$ and $CD8^+$ T cells. To substantiate the findings of this thesis, this characterization should also include a more detailed description of the effect of pH on the species of lactate present in inflammatory exudates. I used unbuffered and bicarbonate buffered growth media to separate the effect of pH from that of the lactate anion (Figure 1). Nevertheless, accurate pH measurements of synovial fluid and determination of its buffering capacity, will deliver potent pathophysiological relevance to the findings.

Lactate is only one of the factors in the inflammatory microenvironment that could influence the function of infiltrating immune cells. It is therefore important to approach this problem from a systems biology perspective and identify other soluble and cellular factors that could potentially interfere with the course of the immune response. Abundance of soluble factors from inflammatory sites can be measured by using for instance non-targeted mass spectrometry analysis, which would allow for an accurate reconstruction of the microenvironment. This will

consequently allow for the identification of the dominant factors that influence T cell infiltration and disease process.

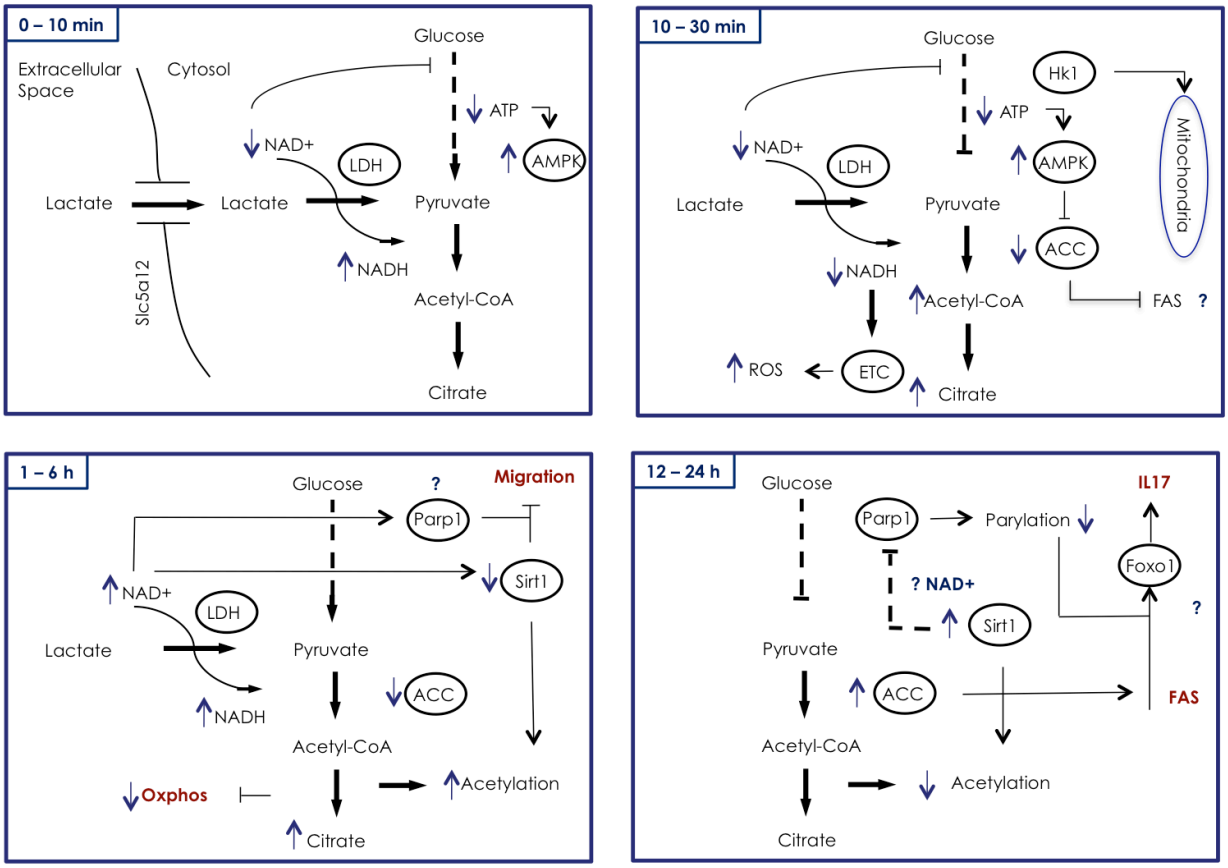

Figure 13 Suggested working model for intracellular lactate signaling in T cells.

Schematic of the suggested time dependent mechanism of intracellular lactate signaling in T-cells

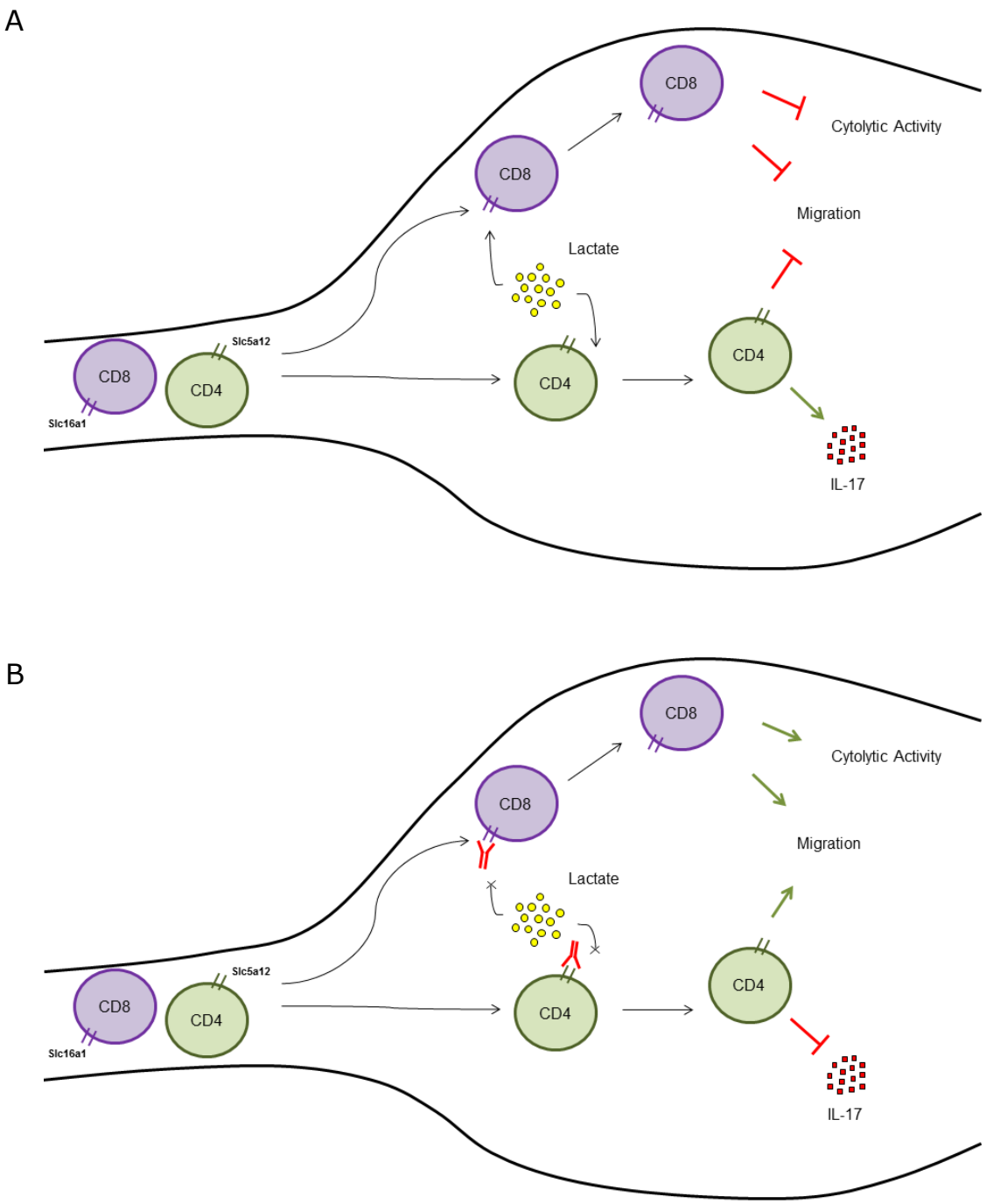

Figure 14 Schematic depiction of the influence of lactate on T cells in the inflammatory site and the effects of lactate transporter inhibition

Schematic of the proposed mechanism of lactate effects on T-cells in the inflammatory site. (**A**) The motility of $CD4^+$ and $CD8^+$ T-cells is blocked once they get exposed to elevated levels of sodium-lactate and lactic acid, respectively, in the inflammatory site. Lactic acid also causes $CD8^+$ T-cells to lose their cytolytic activity and sodium-lactate causes $CD4^+$ T-cells to increase their production of IL-17. (**B**) Anti- lactate transporter antibodies re-establish T-cell migration away from the inflammatory site and block the production of high amounts of IL-17.

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8 APPENDIX

Table 1 Demographical patient data

Parameter	**Study Population *(n=16)***
Age (range)	46-76
Gender (%)	
Female	80
Male	20
Site (%)	
Large joint	68
Small joint	32
Erosive (%)	63
Treatment (%)	
DMARDs	81
Steroids	15
Biologics	61
RF^+ and/or CCP^+ (%)	67

Table 2 List of used qPCR Primers

Primer name	Sequence	Species	Description
Slc16a1_F	GCTGGAGGTCCTATCAGCAG	mouse	Monocaboxylic acid transporter 1
Slc16a1_R	AGTTGAAAGCAAGCCCAAGA	mouse	Monocaboxylic acid transporter 1
Slc5a12_F	AAGCACCTATGAGTACTTACAGC	mouse	sodium-coupled monocarboxylate transporter 2 isoform 1
Slc5a12_R	ACCAGTCACTTGGTTGAGAGC	mouse	sodium-coupled monocarboxylate transporter 2 isoform 1
Hk1_F	AAAGCGGTTCAAAGCCAGTG	mouse	hexokinase-1 isoform HK1
Hk1_R	CACCACAGCTACAATGTTAGCG	mouse	hexokinase-1 isoform HK1
PkM2_F	CCACTTGCAATTATTTGAGGAA	mouse	Pyruvate Kinase M2 Isoform
PkM2_R	GTGAGCAGACCTGCCAGACT	mouse	Pyruvate Kinase M2 Isoform
Glut1_F	CACTGTGGTGTCGCTGTTTG	mouse	Slc2a1 solute carrier family 2, member 1
Glut1_R	ATGGAATAGGACCAGGGCCT	mouse	Slc2a1 solute carrier family 2, member 1
Glut2_F	GGAAGTCAGGGCAAAGAAAAGC	mouse	Slc2a2 solute carrier family 2, member 2
Glut2_R	AATTGGCATCCGTGAAGAGC	mouse	Slc2a2 solute carrier family 2, member 2
Glut3_F	AACTTGCTGGCCATCATTGC	mouse	Slc2a3 solute carrier family 2, member 3
Glut3_R	TGCACAGGCCACAGAAAATG	mouse	Slc2a3 solute carrier family 2, member 3
Glut4_F	TGGCCTTCTTTGAGATTGGC	mouse	Slc2a4 solute carrier family 2, member 4
Glut4_R	AACCCATGCCGACAATGAAG	mouse	Slc2a4 solute carrier family 2, member 4
Pck1_F	TCGGGAAGAAATGCTTTGCG	mouse	phosphoenolpyruvate carboxykinase, cytosolic [GTP]
Pck1_R	TATGCCCAGGATCAGCATATGC	mouse	phosphoenolpyruvate carboxykinase, cytosolic [GTP]
Pck2_F	CCTGGAAACCTGGTGACAAGG	mouse	phosphoenolpyruvate carboxykinase [GTP], mitochondrial
Pck2_R	CTGCTCCCAGAAGTCCTTGG	mouse	phosphoenolpyruvate carboxykinase [GTP], mitochondrial
Fbp1_F	AAAGCCCAAGTGGAAAGCTG	mouse	Fructose-1,6-Bisphosphatase 1
Fbp1_R	ATATCTTTGTCCCCCGTGGTG	mouse	Fructose-1,6-Bisphosphatase 1
Pgc-1a_F	CTCTGGAACTGCAGGCCTAAC	mouse	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pgc-1a_R	CCTTTCTTGGTGGAGTGGCT	mouse	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Ppara	AGCTCACAGAATTTGCCAAG	mouse	peroxisome proliferator-activated receptor alpha
Ppara	TTCCATGATGTCACAGAACG	mouse	peroxisome proliferator-activated receptor alpha
G6Pc_F	AGCTGAACGTCTGTCTGTCC	mouse	glucose-6-phosphatase, catalytic subunit
G6Pc_R	TTCTCCAAAGTCCACAGGAG	mouse	glucose-6-phosphatase, catalytic subunit
Lta_F / TNF-B_F	TGTGTTCCTGCTCAGTAAGGG	mouse	lymphotoxin-alpha precursor (TNFbeta)
Lta_R / TNF-B_R	ACAGTGCAAAGGCTCCAAAG	mouse	lymphotoxin-alpha precursor (TNFbeta)
IL4_F	TCGGCATTTTGAACGAGGTC	mouse	interleukin-4 precursor
IL4_R	TGGTGTTCTTCGTTGCTGTG	mouse	interleukin-4 precursor
IL5_F	CCGCCAAAAAGAGAAGTGTGG	mouse	interleukin-5 precursor
IL5_R	TTCCATTGCCCACTCTGTACTC	mouse	interleukin-5 precursor
IFNg_F	ATCAGGCCATCAGCAACAAC	mouse	Interferon gamma precursor
IFNg_R	TGCATCCTTTTTCGCCTTGC	mouse	Interferon gamma precursor
IL13_F	ATTGCATGGCCTCTGTAACC	mouse	interleukin-13 precursor
IL13_R	GGCGAAACAGTTGCTTTGTG	mouse	interleukin-13 precursor
IL-17_F	AAAGCTCAGCGTGTCCAAAC	mouse	interleukin-17A precursor
IL-17_R	TTCTGGAGCTCACTTTTGCG	mouse	interleukin-17A precursor
Rorc_F	TCAAGTTTGGCCGAATGTCC	mouse	RAR-related orphan receptor gamma
Rorc_R	ACTTGTTCCTGTTGCTGCTG	mouse	RAR-related orphan receptor gamma
SLC16A1_F	CACCCACAGAGGCTTTTTGC	human	monocarboxylate transporter 1
SLC16A1_R	GTCGGGCTACCATGTCAACA	human	monocarboxylate transporter 1
SLC5A12_F	GTGTGCTGTCTTCTCTGGCT	human	sodium-coupled monocarboxylate transporter 2
SLC5A12_R	GCCACAAAAAGTCCTGGCAG	human	sodium-coupled monocarboxylate transporter 2

Table 3 List of used Buffers

Buffer	Composition
Cell Lysis Buffer	50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100
RIPA	50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 1% sodium deoxycholate, 0.1% SDS
Laemmli	0.1% 2-Mercaptoethanol, 0.0005% Bromophenol blue, 10% Glycerol, 2% SDS, 63 mM Tris-HCl, (pH 6.8)
2x BBS	50 mM BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 1.5 mM Na2HPO4, 280mM NaCl
Fixation Buffer	2% paraformaldehyde in 1xPBS
Permeabilization buffer	0.1% Triton X-100 in 1xPBS
FACS buffer	0.5% BSA , 0.05% Azide in 1xPBS
Cell Isolation Buffer	2% FBS, 10mM EDTA in 1xPBS
TBST	0.1% Tween 20 in 1x Tris buffered Saline
Milk	5% non-fat dry milk in 1x TBST

Table 4 List of used antibodies

Antibody	Clone	Source	Producer
CD3	17A2	rabbit	Biolegend
CD28	37.51	rabbit	Biolegend
CD3	Hit3a	rabbit	Biolegend
CD28	28.2	rabbit	Biolegend
Slc5a12	ab107749	rabbit	Abcam
Slc16a1	ab90582	rabbit	Abcam
Hexokinase 1	C35C4	rabbit	Cell Signalling
Pyruvate Kinase M1/2	C5E6	rabbit	Cell Signalling
Aldolase A	D73H4	rabbit	Cell Signalling
Enolase 1	D2S1A	rabbit	Cell Signalling
Pyruvate Dehydrogenase	C54G1	rabbit	Cell Signalling
Acetyl-Lysine	Ac-K-103	rabbit	Cell Signalling
pAMP Kinase α	D5A2	rabbit	Cell Signalling
total AMP Kinase α	40H9	rabbit	Cell Signalling
pAcetyl CoA Carboxylase	D7D11	rabbit	Cell Signalling
total Acetyl CoA Carboxylase	C83B10	rabbit	Cell Signalling
Poly (ADP-Ribose) polymerase 1	10H	rabbit	Adipogen
Histone H3	3H1	rabbit	Cell Signalling
β-Actin	1,30E+06	rabbit	Cell Signalling
CD4	RPA-T4	rabbit	Biolegend
IL17A	BL168	rabbit	Biolegend
Slc5a12	NBP1-92408	rabbit	Novus Biologicals
CD3	17A2	rabbit	ebioscience
CD4	GK1.5	rabbit	ebioscience
CD8	SK1	rabbit	ebioscience
CD25	3C7	rabbit	ebioscience
CXCR3	CXCR3-173	rabbit	ebioscience
CCR7	4B12	rabbit	ebioscience
CD62L	MEL-14	rabbit	ebioscience
LFA-1	YN1/1.7.4	rabbit	ebioscience

Curriculum Vitae

Robert Haas, MSc

Personal data

Adress	58 Princes Square, W24PX Bayswater, London, United Kingdom
Email	r.haas@qmul.ac.uk; haas_robert@gmx.net
Date of birth	31 January 1986
Place of birth	Linz, Austria
Nationality	Austrian

Education

2012 – 2016	PhD FT William Harvey Research Institute (Immunology / Biochemistry - Non-Clinical), Queen Mary University of London, United Kingdom. Investigating the molecular mechanisms of metabolic control of T cell migration (Supervisors: Dr. Claudio Mauro and Prof. Federica Marelli-Berg).
2011 – 2012	Master Studies Biochemistry / Molecular Medicine, University of Vienna, Austria. Targeting CARKL: Drug Design Based On Structure To Function Analysis (Supervisors: Prof. Timothy Skern, Prof. Oswald Wagner, Arvand Haschemi, PhD) Main courses: Biochemistry, Molecular Medicine, Immunology, Structural Biology, Cell Biology
2009 – 2011	Bachelor Studies Biology / Molecular Biology, University of Vienna, Austria. Thesis: "Cancer Stem Cells in the Colon" (Supervisor Dr. Heike Kahr)
2006 – 2009	Bachelor Studies Biotechnology, University of Applied Sciences, Wels, Austria. Exchange semester Athlone Institute of Technology AIT, Athlone, Ireland.

Work experience

2014 – Present	Freelance Academic Writing Consultant for Biological and Medical Sciences; Academic Knowledge, Nottinghamshire, United Kingdom.
2011 – 2012	Master Degree Student; Clinical Institute of Medical and Chemical Laboratory Diagnosis (KILM/KIMCL), Medical University, Vienna, Austria. (Prof. Oswald Wagner and Arvand Haschemi, PhD)
2009 – 2011	Research Associate; Clinical Institute of Medical and Chemical Laboratory Diagnosis (KIMCL), Medical University, Vienna, Austria. Novel Approaches for Fighting Obesity and Diabetes (Prof Oswald Wagner and DDr. Harald Esterbauer)

Gene Targeting of ROSA26 locus in mouse embryonic stem cells to create a novel model for obesity, diabetes and metabolism studies (DDr. Harald Esterbauer and Dr. Emilio Casanova)

Identification of a novel ribonuclease gene family involved in adipocyte differentiation (DDr. Harald Esterbauer and Dr. Martin Bilban)

Characterization of new mediators in adipocyte differentiation (Dr. Martin Bilban)

Additional Training

Jul 2013	Introduction into Statistics in Biology (Queen Mary University of London)
Jan 2013	Laboratory Animal Training Certificate (Queen Mary University of London)
Mar 2012	Bioinformatics and Computational Biology for structural RNA analysis (Prof. Ivo Hofacker, University of Vienna)
Feb 2012	Bioinformatics for Transcriptomics, Meta-Transcriptomics and Functional Genomics (Prof. Thomas Rattei, University of Vienna)
Sep 2011	Structural Biology Course for X-Ray, NMR and Differential Scanning Calorimetry (Gottfried Koehler, Max F. Perutz Laboratories, University of Vienna)
Apr 2011	Gene Targeting of ROSA26 Locus in Mouse Embryonic Stem Cells (Dr. Emilio Casanova, Ludwig Boltzmann Institute for Cancer Research)

Grants, Scholarships & Awards

Oct 2014	Prize for best Poster Presentation, William Harvey Day 2014	£100
Jun 2014	William Harvey Young Investigator Arward	£250
Apr 2013	Grant for Pilot Project; Immunology Research Theme, QMUL	£2000
2012 – 2015	PhD Studentship: Medical Research Council	£15740 pa

Presentations & Conferences

Dec 2014	Oral Presentation at British Socitey for Immunology congress; Bright Sparks in Immunology Session
Dec 2014	Oral Presentation at British Socitey for Immunology congress; Session 'Immunometabolism control in chronic inflammatory diseases'

Patents

Oct 2014	Patent 1418626.6; Targeting Lactate Transporters in chronic inflammatory diseases

Publications

Haas R, Smith J, Rocher-Ros V, Bland EJ, Bombardieri M, Pitzalis C, Marelli-Berg FM, Mauro C. 2015. Lactate Regulates Metabolic and Pro- inflammatory Circuits in Control of T Cell Migration and Effector Functions. *PLoS Biology*.

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Lindroos J, Husa J, Mitterer G, Haschemi A, Rauscher S, **Haas R**, Gröger M, Loewe R, Kohrgruber N, Schrögendorfer KF, Prager G, Beck H, Pospisilik JA, Zeyda M, Stulnig TM, Patsch W, Wagner O, Esterbauer H, Bilban M. 2013. Human but not mouse adipogenesis is critically dependent on LMO3. *Cell Metabolism*.

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Haschemi A, Kosma P, Gille F, Evans C, Burant CF, Starkl P, Knapp B, **Haas R,** Schmid JA, Jandl C, Amir S, Lubec G, Park J, Esterbauer H, Bilban M, Brizuela L, Pospisilik AJ, Otterbein LE, Wagner O. 2012. The Sedoheptulose Kinase CARKL Directs Macrophage Polarization Through Control of Glucose Metabolism. *Cell Metabolism.*